UNIVERSITY OF ALBERTA

THE ROLE OF TUMOR NECROSIS FACTOR ALPHA IN VILLOUS TROPHOBLAST DIFFERENTIATION

By

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ABSTRACT

Proper placental development is required to support the growth of the developing fetus. In intrauterine growth restriction (IUGR), the fetus fails to reach is full growth potential and the placenta may contribute to this pathology. In IUGR, there is an increase in apoptosis in both the syncytiotrophoblast and cytotrophoblast of the villous placenta and an elevation in TNFα in both maternal serum and fetal amniotic fluid. I hypothesized that increased TNFα increases apoptosis and decreases differentiation in IUGR trophoblasts which contribute to decreased placental function. IUGR cultures had higher apoptosis than normal cultures. Contrary to my hypothesis, IUGR cultures had increased differentiation compared to normal trophoblast cultures. This was mediated by endogenous TNFα signaling through both p38 MAPK and ERK1/2 dependent pathways to stimulate both increased syncytialization and increased β-hCG production. Increased apoptosis and differentiation suggests that trophoblast turnover is accelerated in IUGR, leading to decreased placental function.

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION 1

1.0 PROLOGUE 1

1.1 EARLY HUMAN PLACENTAL DEVELOPMENT 1

1.2 THE EARLY VILLOUS PLACENTA 3

1.3 TROPHOBLAST CELL LINEAGES 4

1.3.a Extravillous Trophoblasts 4

1.3.b Villous Cytotrophoblasts 5

1.3.c Villous Syncytiotrophoblast 5

1.4 CHARACTERISTIC FEATURES OF THE TERM PLACENTA 6

***1.5 IN VITRO* MODELS OF PLACENTAL DEVELOPMENT AND TROPHOBLAST DIFFERENTIATION** 6

1.5.a Trophoblast-derived cell lines 6

1.5.b Isolated Primary Trophoblast Cultures 7

1.5.c Placental explants 8

1.6 TROPHOBLAST DIFFERENTIATION 8

1.6.a Structural Features of the Syncytiotrophoblast 9

1.6.a.i Microvilli of the Syncytium 9

1.6.a.ii Involvement of Cell Adhesion Molecules E-cadherin, Cadherin-11 and Beta-catenin in villous trophoblast differentiation 9

1.6.a.iii Involvement of Connexin 43 and Gap Junction Communication in Villous Cytotrophoblast Differentiation 10

1.6.a.iv Phosphatidylserine Externalization and Its Role in Syncytial Fusion 11

1.6.b Functional Features of the Syncytiotrophoblast 12

1.6.b.i Hormone Production by the Syncytiotrophoblast 12

1.6.b.ii Regulation of human chorionic gonadotropin and human placental lactogen expression 13

ii.a) Regulation of Human Chorionic Gonadotropin 13

ii.b.) Regulation of Human Placental Lactogen 14

1.7 FACTORS INFLUENCING TROPHOBLAST FUNCTIONAL AND MORPHOLOGICAL DIFFERENTIATION 15

1.7.a Cyclic Adenosine Monophosphate Stimulation of Trophoblast Differentiation *15*

1.7.b Phosphatidylserine Efflux and The Antiphospholipid Antibody Syndrome *15*

1.7.c The HERV-W Family of Retroviruses-Syncytin *15*

1.7.d The HERV-FRD Env Glycoprotein and Villous Cytotrophoblast Cells *16*

1.7.e Glial Cell Missing (GCMa) and Trophoblast Differentiation *17*

1.7.f The Role of Oxygen in Trophoblast Differentiation *19*

1.8 GROWTH FACTOR MEDIATED TROPHOBLAST DIFFERENTIATION 21

1.8.a Epidermal Growth Factor and Trophoblast Differentiation *21*

EGF and the EGF Receptor 21

Signaling Pathways Triggered by EGF in Trophoblasts 22

1.9 INVOLVEMENT OF PROINFLAMMATORY CYTOKINES IN TROPHOBLAST DIFFERENTIATION 22

1.9.a Interleukin 6 ..... *22*

1.9.b Interleukin-1 ..... *22*

1.9.c Tumor Necrosis Factor-alpha *23*

1.10 TROPHOBLAST TURNOVER AND APOPTOSIS 24

1.10.a Cytotrophoblast Proliferation and Survival *24*

1.10.b Signalling Pathways Involved in Trophoblast Differentiation *25*

1.10.c Trophoblast Cell Death *25*

Necrosis 25

Aponecrosis 26

Apoptosis in Trophoblasts 26

Extrinsic Pathway of Apoptosis: Characteristics of the TNF death receptor family 27

The Intrinsic Pathway of Apoptosis: The Mitochondria and Its Role in Death Receptor-Induced Apoptosis 27

Apoptosis as a Mediator of Trophoblast Differentiation 28

1.11 ABNORMAL PLACENTATION 30

1.11.a Intrauterine Growth Restriction *31*

1.11.b Preeclampsia 32

1.11.c Characteristic Features of Placentae from Preeclamptic/IUGR pregnancies 32

CHAPTER 2: RATIONALE AND HYPOTHESIS 39

2.1 HYPOTHESIS 39

2.2 RATIONALE 40

2.3 EXPERIMENTAL DESIGN 41

CHAPTER 3: MATERIALS AND METHODS 43

3.1 PATIENT POPULATION 43

3.2 VILLOUS CYTOTROPHOBLAST ISOLATION 43

3.3 VILLOUS CYTOTROPHOBLAST CULTURE 44

Thawing of Cryopreserved Stocks and Basic Culture 44

Culture with Peptide Inhibitors 44

Culture with Neutralizing Antibodies 45

Culture with TNFα, EGF and 8-bromo-cAMP 45

Culture at 5% and 2% Oxygen 47

3.4 PREPARATION OF CELL LYSATES FOR WESTERN BLOT ANALYSIS 48

Plating of Cells For Western Blot Analysis 48

Phosphorylation of p38 MAPK in Cytotrophoblasts 48

Phosphorylation of ERK1/2 in Cytotrophoblasts 49

Phosphorylation of JNK in Cytotrophoblasts 49

Syncytin and GCM-1 Expression in Mixed Cytotrophoblast/Syncytiotrophoblast Culture 49

Caspase-8 Cleavage in Cytotrophoblasts 50

Caspase-3 Cleavage in Cytotrophoblasts 51

Electrophoresis and Transfer 51

Blocking and Antibody Incubation 52

3.5 DENSITOMETRY OF WESTERN BLOTS 52

3.6 IMMUNOFLUORESCENT STAINING-DESMOPLAKIN 53

3.7 Immunofluorescent Staining- Placental Alkaline Phosphotase 54
3.8 Immunofluorescent Staining-TUNEL 54
3.9 Immunofluorescent Staining-GCM-1 54
3.10 Visualization of Immunofluorescent Staining, Digital Photography and Analysis 55
3.11 Quantitation of Syncytialization 55
3.12 Quantitation of Apoptosis 56
CHAPTER 4: RESULTS 57
4.1 Patient Population Comprising Study Groups. 57
4.2 Trophoblast apoptosis is increased in IUGR after 5 days under standard culture conditions. 58
4.3 Trophoblast differentiation as assessed by syncytialization, hCG and hPL production is increased in IUGR but not in IUGR-PE. 60
4.4 Elevated trophoblast apoptosis and differentiation in IUGR is blocked by neutralizing antibody to TNFa. 61
4.5 TNFa is able to activate the phosphorylation of p38 MAPK, ERK1/2, JNK and caspase 3 cleavage. 62
4.6 Addition of exogenous TNFa stimulates apoptosis and cell loss in normal and IUGR trophoblasts. 63
4.7 Addition of exogenous TNFa stimulates syncytialization in normal trophoblasts in a dose dependent manner. 65
4.8 Exogenous TNFa suppresses hCG production in both normal and IUGR trophoblasts. 65
4.9 Inhibition of p38a and ERK1/2 MAPKs has differential effects on normal and IUGR trophoblasts. 66
4.10 The pan-caspase inhibitor ZVAD does not inhibit elevated differentiation seen in IUGR. 68

4.11 JNK INHIBITION HAS NO SIGNIFICANT EFFECT ON SYNCYTIALIZATION OR HCG IN NORMAL TROPHOBLASTS. 69

4.12 EXOGENOUS TNFA HAS DIFFERENTIAL EFFECTS ON TROPHOBLAST DIFFERENTIATION MARKERS WHICH ARE MEDIATED BY P38 MAPK AND ERK1/2. 70

4.13 TROPHOBLAST DIFFERENTIATION MARKERS (SYNCYTIALIZATION AND HCG PRODUCTION) ARE DIFFERENTIALLY REGULATED BY CHANGES IN OXYGEN TENSION. 72

4.14 CAMP INDUCES DIFFERENTIATION IN BOTH IUGR AND NORMAL TROPHOBLASTS. 73

4.15 CAMP INDUCES NUCLEAR TRANSLOCATION OF TRANSCRIPTION FACTOR GCM1 IN BOTH NORMAL AND IUGR TROPHOBLASTS. 74

4.16 CAMP UPREGULATES GCM1 BUT NOT SYNCYTIN PROTEIN EXPRESSION. 74

CHAPTER 5: DISCUSSION 100

5.2 TROPHOBLAST APOPTOSIS AND CELL LOSS IS ACCELERATED IN IUGR 100

5.3 TROPHOBLAST DIFFERENTIATION IS ELEVATED IN IUGR TROPHOBLAST CULTURES 101

5.4 TROPHOBLAST DIFFERENTIATION IN IUGR IS MEDIATED BY TNFA 102

5.5 TNFA STIMULATES CASPASE CLEAVAGE AND MAPK PHOSPHORYLATION 102

5.6 ELEVATED DIFFERENTIATION IN IUGR TROPHOBLAST CULTURES REQUIRES ACTIVITY OF ERK1/2 AND P38 MAPKA 103

5.7 EXOGENOUS TNFA HAS DIFFERENTIAL EFFECTS ON TROPHOBLAST DIFFERENTIATION 104

5.8 THE ROLE OF TNFA IN TROPHOBLAST TURNOVER 105

5.9 DIFFERENTIAL EFFECTS OF EXOGENOUS AND ENDOGENOUS TNFA 106

5.10 INTERLEUKIN-1B IS NOT INVOLVED IN TROPHOBLAST DIFFERENTIATION *IN VITRO* 107

5.11 THE CASPASE CASCADE IS NOT INVOLVED IN TROPHOBLAST DIFFERENTIATION *IN VITRO* 108

5.12 IUGR TROPHOBLAST CULTURES ARE RESPONSIVE TO CAMP INDUCED DIFFERENTIATION 109

5.13 THE ROLE OF OXYGEN IN TROPHOBLAST DIFFERENTIATION 110

5.14 MORPHOLOGIC AND FUNCTIONAL TROPHOBLAST DIFFERENTIATION ARE SEPARATE PROCESSES 111

5.15 IUGR AND TNFA: CAUSE OR RESULT? 111

5.16 DOES MODE OF DELIVERY AND SMOKING AFFECT VILLOUS TROPHOBLAST CULTURES? 112

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS **115**

6.1 CONCLUSIONS 115

6.2 FUTURE DIRECTIONS 117

CHAPTER 7: REFERENCES **119**

LIST OF TABLES

Table 3.1. Culture treatments and concentrations.....47

Table 3.2. Antibody suppliers and dilutions.....53

Table 4.1. Patient characteristics of normal, IUGR and IUGR-PE study groups.....59

LIST OF FIGURES

Figure 1.1: Diagram of the utero-placental interface 34
Figure 1.2: Characteristic features of differentiated syncytiotrophoblast. 35
Figure 1.3: Signaling pathways activated by epidermal growth factor (EGF) in trophoblasts. 36
Figure 1.4: Major signaling pathways involved in trophoblast differentiation 37
Figure 1.5: Apoptosis cascade: extrinsic and intrinsic pathways of apoptosis. 38
Figure 3.1: Site of action of peptide inhibitors 46
Figure 4.1. The effect of IUGR on trophoblast apoptosis after 5 days under standard culture conditions 76
Figure 4.2. The effect of pregnancy complications on three differentiation markers (syncytialization, hCG and hPL) in normal, IUGR and IUGR-PE trophoblasts. 77
Figure 4.3. The effect of neutralizing antibody to TNFα on trophoblast apoptosis in normal and IUGR cultures. 78
Figure 4.4. The effect of neutralizing antibodies to TNFα and IL1β on syncytialization, hCG production and number of nuclei remaining in normal and IUGR. 79
Figure 4.5. The effect of TNFα, EGF and p38 MAPK inhibitor SB203580 on p38 MAPK phosphorylation in normal trophoblast cultures (n=3). 80
Figure 4.6. The effect of TNFα, EGF and ERK1/2 inhibitor PD098590 on ERK1/2 phosphorylation in normal trophoblast cultures (n=3). 81
Figure 4.7. The effect of EGF and TNFα on JNK1 and JNK2 phosphorylation. 82
Figure 4.8. The effect of TNFα, ZVAD and cycloheximide on caspase 8 (a, b, and c) and caspase 3 cleavage in normal trophoblast cultures. 83
Figure 4.9. The effect of exogenous TNFα dose on trophoblast apoptosis in normal and IUGR cultures after 5 days of exposure. 84
Figure 4.10. The effect of TNFα dose on normal and IUGR trophoblast survival over a 5 day period. 85
Figure 4.11. The effect of TNFα dose on syncytialization in normal and IUGR trophoblasts. 86

Figure 4.12. The effect of TNFα on hCG production in normal and IUGR trophoblasts. 87
Figure 4.13. The effects of p38 MAPK and ERK1/2 inhibition on normal and IUGR trophoblast syncytialization and hCG production. 88
Figure 4.14. The effects of p38 MAPK and ERK1/2 inhibition on normal and IUGR trophoblast survival over 5 days in culture. 89
Figure 4.15. The effect of pan-caspase inhibitor ZVAD-fmk on trophoblast differentiation and survival in normal and IUGR. 90
Figure 4.16. The effect of inhibition of JNK MAPK with JNK II Inhibitor on trophoblast differentiation (syncytialization and hCG production) and survival (nuclei remaining) in normal (n=4) trophoblast cultures. 91
Figure 4.17. The effect of 1 ng/ml of TNFα on syncytialization (upper panel) and hCG production (lower panel) with and without inhibition of ERK1/2, p38 MAPK and caspase activity. 92
Figure 4.18. The effect of oxygen on desmoplakin defined syncytialization in normal and IUGR trophoblast cultures after 5 days. 93
Figure 4.19. The effect of oxygen on trophoblast differentiation (syncytialization and hCG production) and survival in normal and IUGR after 5 days. 94
Figure 4.20. The effect of 8-bromo-cAMP on hCG production and syncytialization in normal and IUGR trophoblasts. 95
Figure 4.21. GCM-1 immunofluorescent staining in normal trophoblast 4 day culture. 96
Figure 4.22. GCM-1 immunofluorescent staining after 4 days in normal trophoblast cultures induced with 8-bromo-cAMP (10^{-5} M). 97
Figure 4.23. GCM-1 protein expression before and after 8-bromo-cAMP treatment on Day 3 of culture under standard conditions. 98
Figure 4.24. The effect of cAMP on syncytin protein expression in normal and IUGR trophoblast cultures. 99
Figure 5.1. Signaling pathways activated by TNFα to stimulate trophoblast differentiation. 114
Figure 6.1. Overall schematic of TNFα effects in both normal and IUGR villous placentae. 116

ABBREVIATIONS

AIF	Apoptosis-inducing factor
ANOVA	Analysis of variance
APAF	Apoptotic protease activating factor 1
AP-1	Activator protein-1
aPL	Antiphospholipid
ATP	Adenosine triphosphate
Bad	Bcl-2/Bcl-XL associated death promoter
Bax	Bcl-2 associated protein
BCL	B-cell lymphoma/leukemia
BH3	BCL-2 homology domain 3
Bid	BH-3 interacting DD protein
Bim	Bcl-2-interacting mediator of cell death
CAMs	Cell adhesion molecules
cAMP	Cyclic adenosine monophosphate
CBP	cAMP responsive element modulator binding protein
c-fos	c-feline osteosarcoma
cGMP	Cyclic guanine monophosphate
CHX	Cycloheximide
c-myc	c-myelcytomatosis oncogene
CT	Cytotrophoblast
CREB	cAMP responsive element binding protein
Cx	Connexin
DAPI	4,6-diamidino-2-phenylindole
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP binding protein
DISC	Death-inducing signal complex
DMSO	Dimethyl Sulfoxide
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinase
ERV	Endogenous retrovirus
EVT	Extravillous trophoblast
FADD	Fas-associated death domain-containing protein
FBS	Fetal bovine serum
FITC	Fluorescein-5-isothiocyanate
FLIP	Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein
GCM-1	Glial cell missing-1
hCG	Human chorionic gonadotropin
HCMV	Human cytomegalovirus
HERV-W	Human endogenous retrovirus-W
HDAC	Histone deacetylase
HIF	Hypoxia inducible factor
HLA	Human leukocyte antigens
hPL	Human placental lactogen
HUVEC	Human umbilical vein endothelial cells
IAP	Inhibitors of apoptosis
IFN	Interferon
Ig	Immunoglobulin
I-κB	Inhibitor of NF-κB
IKK	I-κB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1RAcP	IL-1R accessory protein
IMDM	Iscove's Modified Dulbecco's medium
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
IUGR	Intrauterine growth restriction
JNK	c-Jun NH_2-terminal kinase

LFA-1	Leukocyte function-associated molecule-1
LH	Leutinizing hormone
LIF	Leukemia inhibitory factor
LPS	Lipopolysacharide
MAC-1	C3 complement receptor
MAPK	Mitogen associated protein kinase
M-CSF	Macrophage colony stimulating factor
Mcl	Myeloid cell leukemia differentiation protein
MEM	Minimum Eagle's medium
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
Mtd	Matador
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor κB
p38	p38-mitogen associated kinase
p53	p53 tumor-suppressor protein
PBS	Phosphate buffered saline
PE	Preeclampsia
PIH	Pregnancy induced hypertension
PI3K	Phosphatidyl-inositol-3-kinase
PKA	Protein kinase A
PLAP	Placental alkaline phosphatase
PS	Phosphatidylserine
RIP	Receptor-interacting protein
SCC	Standard saline citrate
SD	Standard deviation
SEM	Standard error of the mean
SGA	Small for gestational age
Smac	Second mitochondria-derived activator of caspase
ST	Syncytiotrophoblast
TGF	Transforming growth factor

TNFα	Tumor necrosis factor alpha
TRAILR	TNF-related apoptosis inducing ligand receptor
TRADD	TNF-R associated death domain
VEGF	Vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

1.0 Prologue

A normal pregnancy requires an adequate supply of nutrients from the mother to the developing fetus as well as the removal of waste products. The placenta provides this function; it delivers both oxygen and nutrients and removes wastes and serves as the connection between maternal and fetal blood. When nutrient supply is diminished, fetal growth is compromised. This can be due to a number of factors such as decreased blood flow to the placenta but is often due to inadequate placental development which results in insufficient blood supply later in gestation when the placenta can no longer meet the demands of the developing fetus.

Inadequate placental development has been implicated in a number of pregnancy complications such as intrauterine growth restriction (IUGR) and preeclampsia (PE). In addition to inadequate placental development, there is also an environment of inflammation found in these pregnancy complications. This is shown by increased rates of apoptosis in the placenta as well as increased levels of the proinflammatory cytokine tumor necrosis factor alpha (TNFα). Although much is known about apoptosis in the IUGR placenta, specifically in the trophoblast, little is known about trophoblast differentiation in IUGR and if this process is altered by TNFα. In the following introduction, I will discuss normal placental development throughout gestation. Subsequently, key cytokines, growth factors and signaling molecules which play a role in trophoblast differentiation (i.e. the process of cytotrophoblast fusion to form the syncytiotrophoblast) will be discussed. Lastly, the evidence for alterations from normal placental development and a potential role for TNFα in the pathophysiology of IUGR will be described.

1.1 Early Human Placental Development

The majority of human placental structural determinations have been made using aborted tissue from different gestational ages as well as term placenta. Microstructures

were determined using either scanning or transmission electron microscopy of fixed tissue. More recently, immunohistochemical staining of fixed tissue has been done to determine cell type, cell cycle information and cell distribution. Placentae have also been perfused with plastic to allow for determination of villous branching structures and of fetal circulation within the placenta.

Approximately 6-7 days after fertilization, the blastocyst implants in the uterine wall. At this stage, the blastocyst is a flattened vesicle of cells: the cells in the outer layer termed trophoblasts which will develop into the placenta and the fetal membranes and the larger cells on the interior form the embryoblast from which the embryo, umbilical cord and amnion form (13). During blastocyst attachment and after invasion of the underlying endometrial endothelium, the trophoblastic cells at the embryonic pole undergo increased proliferation and result in a double layer trophoblast-the outer of the two being in direct contact with maternal tissue. This layer is called the syncytiotrophoblast (ST) which is formed by fusion of neighbouring cells and the inner layer is termed the cytotrophoblast (CT), which remains unfused and serves as a proliferative source of stem cells for the overlying ST, allowing growth of the trophoblast by continuous proliferation and fusion (13).

Initial studies using electron micrographs of tissue from first trimester spontaneous abortions show that cytotrophoblast proliferation occurs to a much greater extent at the implantation or embryonic pole. As such, this area develops further from the lacunar stage to go on to form the placenta and the remaining trophoblast on the opposing side of the embryo forms the smooth chorion (13). Lacunar formation subdivides the trophoblastic covering of the blastocyst into three distinct layers: 1) the primary chorionic plate, which faces the blastocystic cavity, 2) the lacunar system along with the trabeculae (pillars of ST) and 3) the trophoblastic shell, which faces the endometrium.

The primary chorionic plate is a mostly continuous layer of CT, immediately below which is the lacunar system. The lacunae are vacuoles which are separated from each other by columns of ST (trabeculae). The trabeculae are then invaded by CT from the chorionic plate which subsequently spread over the entire length of the trabeculae, followed by the peripheral ends fusing and forming the trophoblastic shell (the outermost layer of the trophoblast) (13). The invasion into the maternal endometrium, previously

due to the lytic action of syncytial trophoblast, is now due to the proliferative action of the cytotrophoblast, expanding the implantation area (105). This invasive action subsequently triggers the maternal endometrial stromal cells to proliferate and enlarge, forming decidual cells (13). Invasion of maternal endometrium by the trophoblast also erodes the blood vessel walls, leading to leakage of maternal blood and the subsequent maternal perfusion of the lacunar system. Initially, this blood is supplied slowly, only due to capillary pressure, but as further invasion of the trophoblast into the maternal spiral arteries occurs, the blood pressure within the lacunae rises and establishes maternal perfusion of the placenta (13). In humans, efficient maternal blood flow occurs only after the 12th week of gestation (13).

1.2 The Early Villous Placenta

After the presence of maternal erythrocytes within the lacunae, CT proliferation and syncytial fusion in the trabeculae occurs, increasing in length as well as the formation of side branches, which protrude into the lacunae. Once these side branches increase in length and diameter, they are invaded by CT and are termed primary villi (20). Branching of the primary villi increases the surface area for exchange and forms primitive villous trees. The stems which retain contact with the trophoblastic shell are termed anchoring villi. As the branching of the villous trees increases, the lacunar system is transformed into the intervillous space, the main site for nutrient and waste exchange between the mother and the fetus (34).

Recent advances in *in vitro* fertilization as well as ultrasound imaging have expanded knowledge with respect to the structure of the human embryo. Prior to this, the majority of structural determinations of the human placenta were extrapolated from animal models. Between day 18 and 20 post-conception, fetal capillaries begin to form from the mesenchyme. Once capillary development is initiated within the villous stroma, the villi are ranked as tertiary villi. At the same time the allantois, containing fetal vascular structures, merges with the chorionic plate. By the 5th week of gestation, a complete fetoplacental circulation is established (13). The surface area for exchange expands as syncytial sprouts form as a result of local CT proliferation accompanied by syncytial fusion, some being invaded by mesenchymal tissue and developing further with

subsequent branching (13). The placental barrier separates maternal and fetal blood and consists of: 1) a continuous layer of ST covering the villous surface and lining the intervillous space; 2) a layer that is complete (1st trimester) but later becomes discontinuous (2nd and 3rd trimester) of CT; 3) a trophoblastic basal lamina; 4) connective tissue; and 5) fetal endothelium. As the pregnancy progresses, regional thinning of this barrier occurs which allows for greater transport between the mother and the fetus (13).

1.3 Trophoblast Cell Lineages

There are three distinct trophoblast cell lineages, the luminal trophoblast, the extravillous trophoblast and the villous trophoblast, all of which serve different functions in the developing placenta. The latter two lineages will be discussed in more detail in the following section. **Figure 1.1** shows their orientation.

1.3.a Extravillous Trophoblasts

Extravillous trophoblasts (EVT) are populations of trophoblast cells residing outside the placental villi. EVT display two phenotypes: the proliferative and the invasive phenotype. Proliferative EVT are found exclusively in the basal lamina and represent immature EVT stem cells. These cells are characterized by the expression of proliferation marker Ki-67 (26), epidermal growth factor receptor (EGFR) c-erbB-1 (102) and epithelial integrin $\alpha 6\beta 4$ (50). The invasive phenotype EVTs are found deeper in the maternofetal interface and have irreversibly left the cell cycle (**Figure 1.1**). They serve the function of transforming the maternal spiral arteries into a low resistance blood supply to the placenta and continue to invade the maternal tissues continuously throughout the first trimester of pregnancy (34). These differentiated trophoblast express c-erbB-2 (102), interstitial integrin $\alpha 5\beta 1$(50), and exhibit upregulated matrix metalloproteinases (MMP)-2 and 11 (172). Proliferating EVT are present during the first and second trimesters of pregnancy; however, by term EVT display considerably reduced proliferative activities (13). EVTs are thought to secrete angiogenic and vasodilator signals which act to promote local blood flow to the uterus and to mediate the increase in maternal cardiac output and blood volume coupled with the decrease in blood pressure

due to systemic vasodilatation, failure of this signal resulting early-onset intrauterine growth restriction [IUGR] and/or preeclampsia (34). Pregnancies complicated by IUGR and/or preeclampsia are often characterized by shallow EVT invasion of maternal tissue and inadequate transformation of maternal spiral arteries (105).

1.3.b Villous Cytotrophoblasts

Incorporation of H-thymidine into villous CT but not ST and ultrastructural studies indicate CT are precursor stem cells responsible for maintaining the non-proliferating ST by fusing and releasing new cell material (21, 175). During early pregnancy this layer is nearly complete; however, by term this layer becomes discontinuous and can be found beneath only 20% of the ST (13). As pregnancy progresses, the amount of CT present does not decrease but rather as the surface area of the placenta increases the CT layer becomes separated (189). This change is important since, unlike ST and endothelial cells, CT do not express Fc-γ receptors (22). Consequently, transport of immunoglobulins is only possible later in pregnancy when the CT layer becomes fragmented.

As CT differentiate, they exhibit higher levels of free ribosomes, rough endoplasmic reticulum and mitochondria (27, 55, 77, 103). Once the CT reaches an electron density similar to the overlaying ST, initial signs of fusion can be observed (21). Many hormones, growth factors and cytokines have been shown *in vitro* to regulate ST formation and will be discussed in more detail in the following pages.

1.3.c Villous Syncytiotrophoblast

The ST is a single multinucleated cell layer that lines the villous placenta and thus separates maternal blood from fetal tissue (**Figure 1.1**). During earlier stages of pregnancy, the ST is a mostly homogeneous layer with evenly distributed cellular organelles and nuclei indicating no functional differentiation inside this layer, although recently fused regions are still distinguishable from the rest of the ST by the high density of organelles, high enzyme activity, and the unusual large nucleus size (13). However, as the placenta grows and the underlying CT layer becomes asymmetric, the ST begins to undergo structural changes resulting in the formation of highly variable regions with

specialized function yet still remaining a continuous cell layer without border (27, 55, 77, 103). From the 15th week on, regions with differing thickness, composition of organelles and distribution of nuclei can be observed (13).

1.4 Characteristic Features of the Term Placenta

Term placentae are characterized by the presence of large numbers of highly branched villous trees, which provide increased surface area for exchange between the maternal and fetal compartments. As discussed earlier, as pregnancy progresses, there is a thinning of the barrier between maternal and fetal blood, mediated by local loss of underlying CT and a thinning of the overlying ST barrier, providing the optimal situation for gas-exchange (13). The villous trees are lined with fetal capillaries, and as the pregnancy progresses, the capillaries bulge laterally to form multiple gas-exchanging terminal villi that are covered by a very thin layer of ST, termed the 'vasculosyncytial membrane' (34). Fetoplacental angiogenesis (or the formation of new blood vessels and capillaries) is important for successful placental development. As the pregnancy progresses, there is a shift from branching angiogenesis and maturation of vessels into muscularized arterioles and venuoles, towards non-branching angiogenesis and prolapse of peripheral capillaries through the walls of mature intermediate villi to form terminal villi (34). Fetoplacental angiogenesis will be discussed further in Appendix I.

1.5 In Vitro Models of Placental Development and Trophoblast Differentiation

There are several *in vitro* models currently used to elucidate the mechanisms mediating trophoblast differentiation and turnover. Each model has its benefits and pitfalls and care must be taken when extrapolating conclusions made in these models to the *in vivo* placenta.

1.5.a Trophoblast-derived cell lines

The earliest models of trophoblast differentiation used choriocarcinoma derived cell lines such as BeWo, JAR and Jeg-3 cell lines which have been used for close to three decades (13). Advantages of cell lines include ease of culture handling,

immortality and replication in culture. A caveat of choriocarcinoma cell lines is their limitations in signal transduction pathway determination as these cell lines have been created from cancer cells, which always exhibit abnormal signaling especially with regard to apoptosis and survival signaling.

1.5.b Isolated Primary Trophoblast Cultures

As a result of limited meaningful intracellular signaling information available from choriocarcinoma cell lines, several methods have been developed to isolate primary villous CT from normal placentae after the first, second and third trimester of pregnancy. Several methods for isolating CT have been published (41), (69, 75, 118, 158, 212). The most popular method developed by Kliman *et al* involves trypsinization followed by a Percoll density gradient resulting in a population of ~90% villous CT. Percoll however has its disadvantages and may decrease the viability of these cells and this particular method does not produce a large number of cytotrophoblasts. In culture, CT isolated by the Kliman method rapidly differentiate into ST when exposed to fetal bovine serum [FBS] and have much reduced viability by 4 days in culture. In contrast, the Guilbert *et al* method for isolating CT involves trypsin digestion followed by immuno-elimination of non-CT cells using antibody coated glass beads. There are several advantages to this method such as a high yield, leaving cells to be cryo-perserved for repeating experiments, high purity (>99% pure), and extended life in culture over the Kliman method with survival up to 10 to 14 days in medium supplemented with FBS and EGF. These cells also differentiate more slowly than those isolated by the Kliman method, and thus more closely resemble the *in vivo* situation.

Disadvantages to results obtained from primary villous CT cultures are: 1) studies are mainly conducted at atmospheric oxygen tension (20%) [under standard culture conditions], which is not representative of the environment within the placenta (which ranges from 5% to 9%) (13); 2) CT do not proliferate in culture, implying that they have exited from the cell cycle and have begun the differentiation process which may be due to isolation or culture procedures; 3) CT are not alone in the placenta and are juxtaposed against the fetal capillary and basement membrane matrix and are thus in contact with a

milieu of cell types (endothelial, fibroblasts, macrophages, etc) all of which may secrete factors that either stimulate or inhibit trophoblast differentiation.

An additional factor to consider is the separation between morphological (i.e. fusion of CT to form a multinucleated syncytium) and functional (i.e. secretion of β-hCG and hPL) which can be studied in isolated primary villous CT cultures that are much more difficult to distinguish in the third model of villous CT, the placental explant.

1.5.c Placental explants

Placental explant culture involves cutting a small (~2 mg) piece of a villous tree and culturing it suspended on a mesh between the air-media interface. There are several methods and names (such as cultivated trophoblast, placental explant, and placental organ culture) for culturing pieces of placenta (46, 86, 143, 145, 187), but generally the explants can be cultured in atmospheric oxygen and produce β-hCG and hPL after an initial decline following shedding of ST layer damaged during dissection. Several groups have cultured explants at lower oxygen concentrations (46, 163, 164) but care must be taken because culturing explants at 5% oxygen (low normoxic for the placenta) results in an hypoxic interior. Explants offer the advantage of a more accurate approximation of the *in vivo* situation as all of the pertinent cells types are in contact with the CT in the physiological orientation. However, explant data is often hard to interpret as hormone production must be normalized to cell number, a feat nearly impossible with a large sample of tissue. Also, the presence of multiple cell types present in unknown proportion makes the data obtained from intracellular signaling pathways hard to interpret - lower expression of a specific marker may reflect a lower number of CT and not an actual reduction in overall CT expression of that marker.

1.6 Trophoblast Differentiation

Trophoblast differentiation is the process whereby underlying proliferative CT fuse with the overlying differentiated non-growing ST. The ST layer is maintained by CT fusion. Trophoblast differentiation involves two processes: morphological differentiation (fusion and formation of the microvillus border) and functional or

biochemical differentiation (hormone production). Although these processes are initially discussed separately, the factors and signalling molecule intermediates discussed below can stimulate both processes or act in an exclusive manner and stimulate either functional or morphological differentiation. **Figure 1.2** summarizes the major characteristics of differentiated trophoblast.

1.6.a Structural Features of the Syncytiotrophoblast

1.6.a.i Microvilli of the Syncytium

The ST is a giant multinucleated syncytium, lining the entire intervillous space. The apical membrane of the ST, which faces maternal blood, is covered by microvilli, which greatly increases the surface area available for maternal-fetal exchange (13). Along the surface of these microvilli, various enzymes are expressed, such as placental alkaline phosphatase (PLAP) (98), galactosyltransferase, α-amylase, protein kinases, Ca-ATPase, cyclic 3,5-nucleotide phosphodiesterase, and nicotinamide adenine dinucleotide phosphate diaphorase (13). This microvillous surface has been widely studied with respect to expression of immunomodulatory proteins/ligands (170), growth factor receptors: EGF receptor (146), and hormone receptors: LH/CG receptor (67).

1.6.a.ii Involvement of Cell Adhesion Molecules E-cadherin, Cadherin-11 and Beta-catenin in villous trophoblast differentiation

The action of morphological differentiation may be regulated in part by members of the cadherin gene superfamily of calcium-dependent cell adhesion molecules (CAMs) including E-cadherin (127). CAMs are a diverse family of extracellular and cell surface glycoproteins which are involved in cell-cell and cell-extracellular matrix adhesion, recognition, activation, and migration. E-cadherin and β-catenin are members of a family of Ca2+-dependent cell–cell adhesion molecules expressed in cells of epithelial origin and are involved in cell–cell associations (135). Expression of these two molecules can be turned on or off during development, which may mediate cellular morphogenesis which makes them candidates for a role in the differentiation and remodeling of the placenta (135). The cadherins are a gene superfamily and are divided into at least two distinct subfamilies: type 1 and type 2 classical cadherins. The type 1 cadherins includes

E-cadherin and type 2 included human cadherin-11 (73). As villous cytotrophoblasts undergo morphologic differentiation to form ST, there is a decrease in expression of E-cadherin and an increase in cadherin-11 expression which is accompanied by a decrease in β-catenin expression (73). Transfection with a cadherin-11 expression vector caused an upregulation of both morphologic and functional (β-hCG) differentiation in primary trophoblast cultures. When primary villous CT are incubated with antisense oligonucleotides specific for caderin-11, the cells were capable of forming cellular aggregates but did not undergo terminal differentiation and fusion to form syncytium with time in culture, indicating that E-cadherin mediates the aggregation of mononucleate trophoblastic cells, whereas cadherin-11 expression is required for the formation of multinucleated syncytium in primary cell cultures (73). A recent paper has shown an increase in both E-cadherin and beta-catenin in placenta from pre-eclamptic pregnancies, implying that there is increased numbers of undifferentiated CT present in these placentae and that aberrant expression of CAMs may play a role in this disease (135). Others have also shown that E-cadherin expression is increased in pre-eclamptic placental sections, indicating either exaggerated cytotrophoblast proliferation or impaired CT differentiation (25).

1.6.a.iii Involvement of Connexin 43 and Gap Junction Communication in Villous Cytotrophoblast Differentiation

Gap junctions are clusters of transmembrane channels composed of hexamers of connexins (Cx) providing a pathway for the diffusion of ions and small molecules such as cAMP, cGMP, inositol triphosphate, and Ca^{2+} (47). The exchange of molecules through gap junctions is thought to be involved in the control of cell proliferation, in the control of cell and tissue differentiation, in metabolic cooperation and in spatial compartmentalization during embryonic development. It has been shown that blocking gap junction communication with the gap junction uncoupler, heptanol, decreased both the morphological and functional differentiation of primary villous trophoblast cultures (47). Connexins represent a family of closely related membrane proteins, which are encoded by a multigene family that contains at least 20 members in humans (65). Human villous trophoblasts express connexin-43 and is localized to areas between

cytotrophoblastic cells and between cytotrophoblastic cells and the syncytiotrophoblast (65). As connexins make up the gap junction pore and gap junctions were shown to be required for trophoblast differentiation, the authors blocked connexin-43 expression with antisense RNA and found that fusion, hCG production and HERV-W expression were all inhibited (65). This demonstrates that gap junctions, specifically those made of connexin-43, are required for the intracellular signaling necessary for villous trophoblast differentiation.

1.6.a.iv Phosphatidylserine Externalization and Its Role in Syncytial Fusion

Phospholipids are usually maintained in an asymmetric pattern within the plasma membrane of all mammalian cells and are classified into two groups: cholinephospholipids (sphingomyelin and phosphatidylcholine) and aminophospholipids (phosphatidylserine [PS] and phosphatidylethanoalamine) (14). The cholinephospholipids are located in the outer membrane leaflet while the aminophospholipids are located in the inner leaflet (53). The asymmetric distribution of amionphospholipids is maintained by the activity of an Mg/ATP-dependent aminophospholipid translocase which moves externalized aminophospholipids back to the inner leaflet. The translocase is head-group specific and is inhibited by high concentrations of calcium (Ca^{2+}) or vanadate (an inhibitor of Mg2+/ATPase enzymes) (53).

Phospholipid asymmetry is disrupted when PS is externalized on the surface of the cell during apoptosis as well as during intracellular fusion processed such as when myoblasts form myotubules, when sperm and oocyte membranes fuse during fertilization and during villous CT fusion to form ST (53). Several studies have focused on the externalization of PS as a key process in CT fusion to form the ST layer (4, 53, 112). In BeWo cells (a choriocarcinoma cell line used as a model of villous cytotrophoblast), treatment with forskolin (an inducer of adenylate cyclase which upregulates production of intracellular cAMP) stimulated PS externalization, which was blocked by inhibition with vanadate (53). When apoptosis was induced with staurospaurine, PS externalized to the same extent and was blocked by ZVAD-fmk (a pan-caspase inhibitor) (53). ZVAD-fmk however, did not inhibit PS externalization in forskolin treated cells and did not inhibit

fusion (53), suggesting that PS externalization in trophoblast differentiation is independent of caspase activity and is a necessary process in trophoblast differentiation.

Enzymes controlling the location of PS include scramblases and floppases. Scramblases are Ca^{2+} dependent and rapidly transport lipids bidirectionally, without head-group specificity and are able to move a large amount of PS to the outer surface (14, 53). Scramblase activity does not require energy and is resistant to vanadate, but is caspase-dependent and inhibited by ZVAD-fmk (53). Floppase activity is attributable to an Mg^{2+}-dependent ATP binding protein in the family of ATP-binding cassette membrane transporters. Floppase activity is directed outwards and is relatively head-group specific for aminophospholipids, inhibited by increasing intracellular Ca^{2+} concentrations , and is sensitive to inhibition by vanadate (14). Das *et al* show that PS externalization is required for trophoblast fusion and is dependent on a floppase-like enzyme of ABC-transporter family as this process is inhibited by vanadate. PS externalization during apoptosis induced by staurosporine is due to the action of a caspase-dependent scramblase-like mechanism which is inhibited by ZVAD-fmk (53).

1.6.b Functional Features of the Syncytiotrophoblast

1.6.b.i Hormone Production by the Syncytiotrophoblast

The syncytiotrophoblast is the source of two pregnancy related hormones: human chorionicgonadotropin (hCG) and human placental lactogen (hPL). These two hormones are markers of differentiated syncytiotrophoblast as they are not produced by the villous cytotrophoblast (13), however differentiated extravillous trophoblast is also capable of hPL production (74). As cytotrophoblast fusion progresses, the genes responsible for hormone production are activated. Directly before cytotrophoblast fusion, α-hCG is secreted, directly after fusion, β-hCG is secreted in a transitory form between cytotrophoblast and syncytiotrophoblast and finally, hPL is secreted by the syncytial state of the trophoblast (13). Initially, these events were tied to syncytial fusion itself, but *in vitro* evidence suggests that this process is a part of trophoblast differentiation and may be independent of fusion (110). Human CG is composed of a specific β-subunit and an α-subunit common to follicle stimulating hormone [FSH], luteinizing hormone [LH], and thyroid stimulating hormone [TSH] (124).

Regulation of hormone production by the ST remains largely unstudied. However, there is some recent work that shows the complex regulation of two hormones human chorionic gonadotropin beta [β-hCG] and human placental lactogen [hPL]. The hCGβ gene cluster consists of 6 copies, with high sequence similarity to the LH gene. Expression of *hCGβ* is mainly controlled at the mRNA level with the promoter region being very complex and known to interact with broadly expressed transcription factors from the selective promoter factor (Sp) and activating protein 2 (AP-2) families (124). Stimulation of hCG production by treatment with forskolin resulted in increased binding of the transcription factor AP-2 to the *hCGβ* promoter (124). Although little further knowledge regarding regulation of *hCGβ* expression is available, this important molecule in trophoblast differentiation must be studied further to understand its role in placental development.

Although β-hCG release is a marker of differentiated ST, it is still unclear if hCG itself modulates trophoblast differentiation. Cytotrophoblasts *in vitro* fail to differentiate both functionally and morphologically when antibody to hCG is added (57), indicating a role for hCG in the differentiation process itself. Conversely, others have shown that hCG is required for morphological differentiation only when stimulated by EGF, LIF or TGF-α, but cAMP induced syncytialization is not inhibited by neutralizing antibody to hCG (209).

1.6.b.ii Regulation of human chorionic gonadotropin and human placental lactogen expression

The acute control of hPL and hCG secretion from the ST layer into the maternal blood stream remains poorly understood despite years of study (127). *In vivo*, there is no known ligand which modulates the release of hPL and/or hCG from the placenta, although *in vitro*, influx of Ca^{2+} into the ST results in an immediate and sustained increase in both hPL and hCG release (12, 169).

ii.a) Regulation of Human Chorionic Gonadotropin

Recent evidence suggests that human chorionic gonadotropin (hCG), in addition to its well-known endocrine effects on the corpus luteum, may act as a growth and differentiation factor during pregnancy (138). The production of hCG is a temporally

regulated process. At the initiation of pregnancy, its exponential secretion and long circulatory half-life serve to extend the life span of the corpus luteum in order to maintain the supply of progesterone during the first 6-8 weeks of pregnancy (193). Stimulation of the human endometrium in the luteal phase of the menstrual cycle with an intrauterine microdialysis system induced the production of paracrine mediators of differentiation (insulin-like growth factor binding protein and prolactin) and implantation (leukocyte inhibitory factor [LIF], macrophage colony stimulating factor [M-CSF]) as well as the production of vascular endothelial growth factor [VEGF], an important cytokine involved in angiogenesis which implicates a role for hCG in both endometrial vascularization and placentation (138).

In addition to the temporal regulation of hCG secretion, there is also temporal regulation of the hCG/leutinizing hormone [LH] receptor on the surface of the trophoblast itself. Before the ninth week of gestation, villous CT and ST express a truncated form (50 kDa) of the hCG/LH receptor, rendering these cells likely unresponsive to hCG signalling (138). However, later in gestation, trophoblasts express the full length (80 kDa) receptor which allows for the potential for hCG to modulate the differentiation of trophoblasts (138). Indeed, hCG signal transduction activates protein kinase A (PKA)-independent phosphorylation of extracellular signal regulated kinase (ERK 1/2) in baboon endometrial epithelial cells (193), activation of which has been shown to be involved in trophoblast differentiation (51).

ii.b.) Regulation of Human Placental Lactogen

Human placental lactogen (also known as human chorionic somatomammotropin) expression has not been extensively studied and much remains unknown. However, a small number of reports exist in the literature. The promoter region for hPL contains a putative AP-1 site and three nuclear transcription factor NF-IL-6 binding sites, suggesting a role for interleukin-6 [IL-6] or inflammation itself in hPL expression (113, 195).

1.7 Factors Influencing Trophoblast Functional and Morphological Differentiation

1.7.a Cyclic Adenosine Monophosphate Stimulation of Trophoblast Differentiation

The intracellular signalling molecule cyclic adenosine monophosphate (cAMP) was one of the first molecules implicated in inducing trophoblast differentiation *in vitro*, stimulating both morphologic and functional differentiation when intracellular cAMP levels rise (110, 118). Since the discovery that cAMP levels rise during trophoblast differentiation, cAMP has been extensively used to stimulate CT differentiation *in vitro*. The actions of cAMP in this process are mediated by the cyclic-AMP dependent protein kinase (PKA) (114). There are two forms of PKA, type I and type II, which differ in their cAMP-binding regulatory subunits (RI and RII) but have similar catalytic subunits, and both of these forms are expressed in the placenta (114). Cyclic-AMP has been studied with respect to every process/marker of trophoblast differentiation including alpha- and beta-hCG production (125, 166, 180) , hPL production (78), downmodulation of desmoplakin (114) and E-cadherin expression (43), upregulation of connexion 43 expression (122), externalization of phosphatidylserine (53), and enhancing ezrin expression in ST microvilli and microvilli morphogenesis (114).

1.7.b Phosphatidylserine Efflux and The Antiphospholipid Antibody Syndrome

Antiphospholipid (aPL) antibody syndrome results from the production of autoantibodies against negatively charged phospholipid dependent antigens in particular cardiolipin and phosphatidylserine (4). The aPL antibody syndrome is associated with high pregnancy loss rates (50-90 %), placental dysfunction, intrauterine growth restriction (IUGR) and pregnancy induced hypertension (PIH) (4, 112). Antibodies directed against PS inhibited fusion of cytotrophoblast-like JAR cells, inhibited invasion of first trimester trophoblasts and inhibited hPL and hCG production in term trophoblast cultures (4, 112).

1.7.c The HERV-W Family of Retroviruses-Syncytin

In 1999, a previously uncharacterized human endogenous retrovirus family, the HERV-W family, was described at the molecular and phylogenetic level (18). Although

HERV-W is not expressed as a complete provirus, its envelope is expressed in the placenta and in trophoblast-derived cell lines (121). The envelope gene of HERV-W was further characterized to be expressed as two major transcripts of 4 and 8 kilobases in length, the mature envelope protein consisting of 518 amino acids and is thought to be a membrane protein based on its amino acid sequence (19, 152). The mature protein associates with an amino acid transporter/retrovirus receptor (the sodium-dependent neutral amino acid transporters ASCT2 and ASCT1) and due to the stimulation of cell-cell fusion processes, it was named syncytin. Syncytin 1 is capable of inducing syncytialization (morphological differentiation) and β-hCG production (functional differentiation) when overexpressed in transfected BeWo cells (152) and these effects can be blocked *in vitro* by antibodies directed against syncytin or antisense RNA to syncytin (152), (66).

Syncytin 1 expression has been localized to all types of trophoblastic cells, both villous and extravillous cell types (142). Syncytin 1 is post-translationally cleaved into a surface (SU) subunit and a transmembrane (TM) subunit which contains a fusion peptide. Current dogma suggests that syncytin 1 binds to the receptor via its SU subunit, which results in a conformational change in the TM subunit, mediated by two repeat regions within the TM subunit, in the fusion process (37).

1.7.d The HERV-FRD Env Glycoprotein and Villous Cytotrophoblast Cells

Recently, a second HERV gene has been identified, HERV-FRD or syncytin 2 (17). In contrast to syncytin 1, its expression is localized to the villous cytotrophoblast and is not expressed in the syncytiotrophoblast or extravillous trophoblast (17). Syncytin 2 is an older retrovirus than syncytin 1, being integrated into the primate genome earlier and is found in both Old World and New World Monkeys as well as humans. In contrast, syncytin 1 is uniquely hominoid in expression (28). Cellular localization of syncytin 2 is not solely restricted to the cell membrane, as it is also found in the cytoplasm, but the majority of staining is along the boundary of the cell (17), implying a role in mediating the fusion process. Further characterization of syncytin 2, including its cellular receptor, is required before any further conclusions can be drawn regarding its role in cytotrophoblast fusion and differentiation.

1.7.e Glial Cell Missing (GCMa) and Trophoblast Differentiation

Glial cell missing (GCM)-1 was first isolated from a *Drosophila melanogaster* mutant that produces additional neurons at the expense of glial cells (37). GCM-2 has recently been identified as a homologue of GCM-1 and although it plays a minor role in gliogenesis, it is required (along with GCM-1) for proper differentiation of plasmatocyte/macrophage cell line (37). Two homologs, GCMa and GCMb have been identified in mice, rats and humans (37, 40). All members of the GCM family so far identified are key regulators of differentiation processes (122). In mice, GCMa and GCMb regulate the formation of placental syncytiotrophoblasts and parathyroid gland, respectively (40). GCMa knockout mice have a complete lack of branching at the chorioallantoic interface and no fusion of trophoblast cells to syncytiotrophoblast cells (39), which led to mid-embryonic death due to failure to form a functional placenta (122). In mouse, GCMa mRNA is highly expressed in the labyrinthine trophoblast cells of the placenta and at low levels in restricted sites in the post natal kidney and thymus (37).

GCM proteins form a new family of transcription factors and possess a conserved zinc-coordinated DNA binding domain, the GCM motif, at the amino terminus (37), (122). The DNA binding domain is made up of two subdomains each comprised of beta sheets which interact with the major grove of its cognate DNA element 5'-ATGCGGGT-3' (122), (37). The first target of GCMa identified in humans was syncytin, the fusigenic glycoprotein originally derived from a human endogenous retrovirus HERV-W, which is highly expressed in the ST layer of the placenta. GCMa is able to regulate syncytin gene expression via two GCM-1 binding sites upstream of the 5'long terminal repeat of HERV-W in choriocarcinoma cell lines (211).

When GCM-1 mRNA and protein expression was analysed in the human placenta over the course of gestation, it was found to be expressed in clusters of villous cytotrophoblasts which tended to be uniformly spaced along the surface of the villi (8). GCM-1 expression was not found in villous CT at the base of cell columns, which serve as the precursors of the invasive extravillous CT (8). Cellular localization of GCM-1 was mainly nuclear in CT nuclei but both nuclear and to a lesser extent cytoplasmic in ST nuclei, implying GCM-1 is expressed both before and after CT fusion to ST (8).

In vitro studies using both isolated primary trophoblasts and immortalized choriocarcinoma cell lines have shown that syncytin expression is upregulated by cAMP and forskolin and down regulated by low oxygen (hypoxia treatment of 1 to 3 percent oxygen) (122). However, cAMP or forskolin prevented the hypoxia induced decline in syncytin expression (122). In vitro studies using both isolated primary trophoblasts and the immortalized choriocarcinoma cell line BeWo have shown an upregulation of GCMa mRNA expression after treatment with forskolin or cAMP and a down regulation following exposure to hypoxia (122). In BeWo cells, forskolin and to a lesser extent cAMP partially reversed the hypoxia induced decline in GCMa mRNA but in primary trophoblasts cultured in hypoxia (1 to 3 % oxygen), cAMP agonists counteracted the loss in GCMa mRNA to a lesser extent compared to cells cultured under normoxia (8% oxygen).

When the protein kinase A (PKA) catalytic subunit was transfected into BeWo cells, there was an increase in GCMa mRNA and an eight fold increase in GCMa protein activity, demonstrating that PKA activity increases GCMa expression and increases cell differentiation (as PKA transfection was accompanied by an increase in connexin 43 expression) (122).

Other authors have shown that GCMa specifically interacts with CREM (cAMP responsive element modulator) binding protein [CBP] and this interaction was enhanced when PKA activity was increased (37) in BeWo cells. These authors also showed that cAMP/PKA signaling pathway activation by forskolin leads to increased association of GCMa and CBP with the syncytin promoter and an accompanying increase in syncytin gene expression. This effect is independent of p38 or ERK1/2 MAPKs as inhibition with SB203580 or PD098059 did not alter the effect of forskolin on GCMa activity (37). The interaction of CBP and GCMa allows CBP to acetylate *GCMa*, which increases the GCMa protein stability by blocking GCMa ubiquitination (37).

CBP (a transcription coactivator and histone acetyltransferase [HAT]) acetylation of GCMa stabilized the transcription factor, but a recent paper has shown that a specific histone deacetylase (HDAC3) reverses GCMa acetylation and can attenuate CBP-upregulated GCMa activity (40). This deacetylase (HDAC3) binds to the promoter of the

syncytin gene but becomes disassociated once BeWo cells are stimulated with forskolin (40).

Given the complex regulation of syncytin gene expression by multiple factors, including cAMP, GCMa, CBP, and HDAC3, and its key role in trophoblast morphologic and functional differentiation, syncytin and GCM1 have been studied with respect to preeclampsia, which is often accompanied by abnormal trophoblast invasion and deficiency of terminal villi (39). In preeclampsia, syncytin is dramatically reduced and the protein itself is abnormally localized to the apical ST microvillous membrane (130), (120). GCMa mRNA expression was also lower in preeclamptic placenta compared to gestational age matched normal controls (39), although whether this is due to a decrease in the number of CT/ST in the sample of the placenta used was not accounted for in this study. Although IUGR was not included in this study, it leads to speculation that GCMa expression may also be lower in IUGR placenta as IUGR placentae are characterized by abnormal placentation.

1.7.f The Role of Oxygen in Trophoblast Differentiation

Initial studies using human trophoblast cell lines and primary human trophoblasts were done under standard culture conditions at atmospheric oxygen (~18% oxygen or 138 mmHg). However, during placental development, the trophoblast is exposed to a varied range of oxygen tensions. In early pregnancy (8 to 10 weeks gestation), before the extravillous trophoblast has invaded the maternal endometrium, blood flow to the placenta is minimal and the trophoblast is exposed to oxygen tensions between 18 to 40 mmHg (179). Once endovascular invasion proceeds, CT are in direct contact with blood from the maternal spiral arterioles which may have oxygen tensions as high as 90 to 100 mmHg (72). Indeed, trophoblasts cultured under low oxygen tensions (~15 mmHg or 2% oxygen), mimicking the environment of early pregnancy, can be stimulated to proliferate (72). In contrast, those cultured at 6% oxygen (~45 mmHg) showed the same low numbers of proliferating nuclei as those cultured under standard conditions (72). Other authors have shown that low oxygen tensions (hypoxia or 3% oxygen) decrease the expression of syncytin and in turn decrease differentiation markers in BeWo cells (122). This suggests that an environment of low oxygen (~15 mmHg) stimulates trophoblast

proliferation as seen in early pregnancy and as oxygen tensions rise with increasing EVT invasion, CT are stimulated to differentiate into ST.

In mammalian cells there is an adaptive response to hypoxia that is accompanied by increased expression of a number of genes including hematopoietic growth factor, VEGF, glycolytic enzymes, and inducible nitric oxide synthetase (64, 71, 151, 185). Most of these genes are regulated by a common oxygen-sensing pathway involving the formation of the hypoxia-inducible factor-1 (HIF-1) protein complex. HIF-1 is a helix-loop-helix transcription factor able to bind to a short DNA motif in the 5′ flanking regions of hypoxia-induced genes. There are two isoforms of HIF-1 which are differentially expressed, HIF-1α being inducible by hypoxic conditions and degraded under normoxic conditions and HIF-1β being constitutively expressed. Recently, HIF-1α has been shown to mediate trophoblast differentiation through regulation of TGFβ expression, TGFβ being expressed during early pregnancy in low oxygen conditions and acts to suppress trophoblast differentiation (31). More recently, HIF-1α and HIF-2α knockout mice have shown a requirement for oxygen sensing in trophoblast lineage determination, the knockouts showing placentas that are avascular and have poor decidual invasion, reduced spongiotrophoblast numbers (CT equivalent), expanded trophoblast giant cell (ST equivalent) and defective labyrinthine trophoblasts (murine EVT equivalent) (44).

In IUGR pregnancies, there is evidence for inadequate delivery of oxygen to the fetus either due to delivery to the placenta or delivery across the placenta or a combination of both (117). Blood gas analysis of the intervillous space and umbilical vein samples from IUGR placentae show wider intervillous-umbilical gradients of oxygen tensions than normal placenta with higher intervillous levels and lower umbilical vein levels (167). As oxygen plays such an important role in trophoblast differentiation, the wider range of oxygen tensions that CT are exposed to could contribute to the pathophysiology of IUGR (115).

Further *in vitro* work has shown that lower oxygen tensions impair trophoblast differentiation by decreasing hCG production and syncytialization (126). In addition to preventing differentiation of CT and promoting proliferation, the low oxygen tensions of early pregnancy also protect the placenta from oxidative stress. As oxygen tension rises

throughout gestation, the ST and CT are exposed in higher concentrations of oxygen, leading to the production of reactive oxygen species and the induction of apoptosis. In IUGR, the higher levels of intervillous oxygen may lead to increased oxidative stress and contribute to the elevated apoptosis seen in this disease (160).

1.8 Growth Factor Mediated Trophoblast Differentiation

A variety of growth factors have been shown to modulate trophoblast differentiation, both morphological and functional. Increasing evidence indicates that syncytium formation is an autocrine- and paracrine-regulated event involving a number of trophoblast and nontrophoblast products in the placenta (209). Products shown to increase differentiation of trophoblasts include epidermal growth factor (EGF), leukemia inhibitory factor (LIF), estradiol/estrogen, granulocyte macrophage colony stimulating factor[GM-CSF], colony stimulating factor [CSF-1], fibronectin, collagen I, glucocorticoids [dexamethasone] and hCG and inhibited by transforming growth factor beta [TGF-β] (156, 209). Although multiple growth factors have been shown to be involved in trophoblast differentiation, I will focus my discussion on EGF, as EGF and EGF receptor expression have been shown to be lower in IUGR (29, 204) as well as in preeclampsia (128).

1.8.a Epidermal Growth Factor and Trophoblast Differentiation

EGF and the EGF Receptor

EGF is able to promote proliferation and differentiation as well as being a potent inhibitor of apoptosis (70, 104, 206). EGF is synthesized as a glycosylated transmembrane protein (104) that becomes mature and soluble after proteolysis (206). The target of the EGF family of growth factors (which includes TGF-α, heparine-binding EGF-like growth factor [HB-EGF] and amphiregulin) is the EGFR family of receptor tyrosine kinases (RTKs) consisting of EGFR, HER2/ErbB2/neu, HER3/ErbB3 and HER4/ErbB4 (33, 42, 100). Once EGF binds to its receptor, it induces the formation of homo-or heterodimers between EGF family members, leading to the autophosphorylation of cytoplasmic tyrosine residues (174). In trophoblasts, the presence of ErbB1 (EGFR)

and ErbB3 have been localized to both CT and ST in the rabbit and human (1, 102, 119) and other EGF receptor family members have been shown to be expressed on cells of epithelial origin (207).

Signaling Pathways Triggered by EGF in Trophoblasts

Upon ligand binding, the EGFR exerts the majority of its biological function via its tyrosine kinase activity, the first step in EGFR-mediated signal transduction (104). The downstream activation of a variety of signaling pathways, most of which help ensure survival of the cell, are stimulated by EGFR-ligand binding and include activation of: all of the major mitogen-associated protein kinases (MAPKs) [p38 MAPK, extracellular signal-related kinase [(ERK)-1 and -2, and c-Jun NH2 terminal kinase (JNK) (174); the Src family of kinases (139); Janus Activated Kinase/Signal Transducers and Activators of Transcription [JAK/STAT] pathway (186); the phosphatidyl-inositol-3-kinase [PI3K) pathway (192); and phospholipase Cγ (38). **Figure 1.3** summarizes the pathways stimulated by EGF binding to EGFRs.

1.9 Involvement of Proinflammatory Cytokines in Trophoblast Differentiation

1.9.a Interleukin 6

Interleukin-6 (IL-6) is produced by many different cell types and has a wide variety of physiological functions, including the stimulation of hormone production. In the placenta, macrophages and ST produce IL-6, which stimulates the production of hCG and hPL (113, 195). The villous trophoblast (CT isolated by the Kliman method) expresses IL-6 receptors (an 80 kDa protein) (147), which allows IL-6 to have an autocrine action on trophoblast function (109, 165).

1.9.b Interleukin-1

Interleukin-1 (IL-1) is member of the pro-inflammatory cytokine family secreted mainly by mononuclear phagocytes but also by fibroblasts, keratinocytes, and vascular endothelial cells (58). There are two forms of IL-1, α and β, which are translated from two different regions of the genome and are initially synthesized as 31 kDa proteins.

There is a third, structurally related molecule, IL-1 receptor antagonist (IL-Ra), which competes with IL-1 for binding sites on cell surface receptors (59). In contrast to IL-1β, IL-1α remains within the cytoplasm and is released only from dead or dying cells, IL-1β being transported out of the cell and is cleaved to its 17 kDa extracellular form. Two IL-1 receptors have been identified, IL-1R1 and IL-1R2, binding to IL-1R1 induces a variety of signaling cascades, and binding to decoy receptor IL-1R2 results in a lack of signal transduction (68). IL-1R1 has been localized to the ST and expression of IL-1β has been shown in villous CT and ST as well as the intermediate trophoblast and maternal stromal decidual cells (188).

Expression of IL-1β is higher in first trimester placenta than at term (111). IL-1β has been shown to down regulate expression of E-cadherin, ezrin and beta-catenin in both primary villous CT and JEG-3 cells (111), all of which are molecules associated with villous CT, indicating a role for IL-1β in trophoblast differentiation. IL-1 stimulates hCG release via an upregulation of IL-6 (113, 195). More recently, IL-1β has been shown to induce IL-6 and hCG production in a NFκB dependent manner (200).

1.9.c Tumor Necrosis Factor-alpha

Tumor necrosis factor (TNF)α is a proinflammatory cytokine with a diverse range of biological activities. TNFα has multiple effects that include cell proliferation, cell death and the activation and inhibition of cellular functions. The net biological effects of TNFα can be either detrimental or beneficial to the cell depending on the amount of TNFα present, the duration of cell exposure and the presence of other biological mediators that synergize or antagonize TNFα activity (30).

The active form of TNFα is a trimer, consisting of three 17 kDa subunits arranged in a triangular pyramid (76). The secreted form of TNFα is able to bind to either of its two receptors: TNFR1 (p55) and TNFR2 (p75). Upon binding of its receptor, TNFα is able to mediate a wide range of cellular responses ranging from survival signaling via activation of MAPKs to apoptosis via activation of the caspase cascade (155). **Figure 1.5** shows the major signaling pathways activated by TNFα through TNFR1.

Villous trophoblast express TNFR1 (p55 receptor) (214) and are highly susceptible to the effects of the inflammatory cytokine TNFα, a major inducer of

apoptosis (70, 213). Although TNFα is found in normal healthy placental tissue and is produced by trophoblast (137, 210), suggesting a role in normal placental development (155), it is also clear that aberrant expression may lead to pathological placental conditions resulting in fetuses displaying intrauterine growth restriction (IUGR). Placentae isolated from rat and human pregnancies complicated with IUGR showed significant increases in the rate of apoptosis (61, 153, 191). Women with IUGR pregnancies had higher serum levels of TNFα (11) and IUGR fetuses have elevated TNFα in their amniotic fluid (83). IUGR human placentae showed increased TNFα production (85) and increased susceptibility to TNFα (45). Elevated levels of TNFα in the placenta of IUGR pregnancies may lead to elevated apoptosis, whereas normal, physiological levels of TNFα may play a role in the stimulation or modulation of trophoblast differentiation in normal pregnancies (155).

1.10 Trophoblast turnover and apoptosis

Trophoblast turnover, in simple terms, is the life cycle of the CT as it progresses from fusion into the ST, exerts its biological function as part of the syncytium and then, as the nucleus ages, undergoes apoptosis and is packaged into syncytial knot structures with other apoptotic nuclei and then shed into the maternal blood stream. Turnover is the balance between the proliferation of CT, fusion of underlying CT and differentiation into functional ST and shedding of apoptotic nuclei in syncytial knots (103). However, trophoblast turnover is multifaceted due to the complexity of the regulatory mechanisms involved in the control of CT proliferation, survival, ST differentiation and ultimately apoptosis.

1.10.a Cytotrophoblast Proliferation and Survival

Little is known about the control of the cell cycle in villous trophoblasts. In early pregnancy, proliferation of the trophoblast is high and falls as the pregnancy approaches term (54). Proliferation of trophoblast may be stimulated by hypoxia (31), fibroblast

growth factor [FGF]-4 (7), EGF, transforming growth factor alpha [TGFα], insulin-like growth factor 1 [IGF-1] and inhibited by TGFβ (136).

Trophoblast survival has been shown *in vitro* to be mediated by EGF stimulation of fibronectin production (171) through unknown signaling intermediates and by placental growth factor through stimulation of p38 and JNK MAPKs (56). In addition to EGF, heparin (84) and heparin-binding EGF have recently been shown to stimulate trophoblast survival through activation of multiple anti-apoptotic signaling pathways including PI3K, JNK, and ERK1/2. At low oxygen tension, such as that found in the first trimester of pregnancy, heparin-binding EGF is also able to mediate trophoblast survival (6).

1.10.b Signalling Pathways Involved in Trophoblast Differentiation

A variety of complex and interwoven signalling pathways have been shown to be involved in CT differentiation and fusion. The transcription factor GCMa (211), the proinflammatory cytokines IL-6 and IL-1β (195, 200), the hormone β-hCG (209), the protein syncytin (173) as well as intracellular signalling molecules cAMP (166), p38 MAPK (101), ERK1/2 (51), and Src family kinases (52) have all been shown to be involved. **Figure 1.4** shows known signalling pathways involved in trophoblast differentiation.

1.10.c Trophoblast Cell Death

The death of a cell may be accidental (necrosis) or an active process (apoptosis). Both types of cell death are seen in the placenta, but apoptosis is thought to be the major mediator of placental development.

Necrosis

Necrotic cell death occurs in cells that are damaged by injury such as by mechanical damage or exposure to high doses of toxic chemicals, and is characterized by

cellular, nuclear and mitochondrial swelling due to permeablization of the plasma membrane, resulting in cellular rupture and leakage into surrounding tissues (92).

Aponecrosis

Recently, an intermediate form of cell death has been characterized that demonstrates features of both apoptosis and necrosis and is termed "aponecrosis". In this type of cell death, apoptosis is initiated but fails at some point along the signaling pathway, resulting in a switch to necrosis to complete the death of the cell (63).

Apoptosis in Trophoblasts

As the placenta develops, it undergoes constant tissue remodeling, characterized by the functional loss of trophoblast cells by the action of apoptosis (197). Apoptosis, or programmed cell death, is an active process by which cells are eliminated to maintain normal tissue function. Apoptosis is also associated with characteristic morphological changes, including cell shrinkage, release of cytochrome c from the mitochondria preceded by mitochondrial depolarization, membrane blebbing and nuclear chromatin degradation (92). Unlike necrotic cell death, which stimulates inflammation as cytoplasmic contents are released into the surrounding tissue, apoptosis culminates in the release of small, membrane wrapped fragments termed apoptotic bodies which are engulfed by phagocytic cells which then secrete cytokines to inhibit inflammation (92).

Apoptosis can be initiated by one of two known pathways: intrinsically by the mitochondrial pathway and extrinsically by either the death receptor-mediated pathway or in response to exogenous stimuli such as cytokines (183). **Figure 1.5** shows the major steps in both signaling cascades. Caspases are the main effectors of both extrinsic and intrinsic pathways of apoptosis. Caspases are intracellular proteases that use cysteine as the nucleophilic group for substrate cleavage, cleaving the peptide bond C-terminal to aspartic acid residues (92). As with most other cellular proteases, caspases are synthesized as inactive precursor "procaspases" which remain inactive until stimulated (197). There are two groups of caspases: 1) caspases with long prodomains (caspase-2,-8,-9, and -10) that act as initiators of apoptosis by cleaving and activating the second group 2) caspases with short prodomains (caspase-3,-6,-7, and -14) that act as

downstream effector caspases (92). Activation of the caspases can lead either directly to the induction of apoptosis or to the induction of mitochondrial membrane permeablization, leading to the release of pro-apoptotic cytochrome c [cyto-c] as well as second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein (DIABLO), which facilitates caspase activation by inhibiting proteins from the inhibitors of apoptosis (IAP) family, into the cytoplasm (199).

Extrinsic Pathway of Apoptosis: Characteristics of the TNF death receptor family

An external stimulus can initiate apoptosis via the extrinsic pathway, mediated by members of the TNF death-receptor family, all of which contain a C-terminal region known as the death domain (96). Thus far, eight members of this family have been identified, including Fas, TNFR1, and TNF-related apoptosis inducing ligand receptor [TRAILR]1 and R2, all of which are expressed by the trophoblast (197). Upon binding of a death inducing ligand (i.e. TNFα) to its membrane-bound death receptor (i.e. TNFR1), there is an activation of pre-associated death receptor trimers. In order for transduction of the death signal, TNF death receptors have intracellular death domains which mediate interactions with death domain-containing adaptor proteins such as Fas-associated death domain (FADD) and TNF-R associated death domain (TRADD) (197). Once the death domain (FADD or TRADD) associates with the receptor, it recruits other cellular proteins including procaspases-8 and -10 via death effector domains (DED) to form the death-inducing signal complex (DISC), after which the initiator procaspases can be activated to caspase 8 and 10 (197). Upon activation of initiator caspases, downstream effector caspases-3,-6, and -7 are activated, eventually culminating in the death of the cell (197).

The Intrinsic Pathway of Apoptosis: The Mitochondria and Its Role in Death Receptor-Induced Apoptosis

In the intrinsic pathway, as the name implies, the death inducing signal originates from within the cell itself, specifically within the mitochondria. In response to cellular stresses, such as DNA damage or growth factor deprivation, the mitochondrial apoptotic pathway can be activated by p53-a tumor suppressor protein that transactivates pro-

apoptotic B-cell lymphoma/leukemia (BCL)-2 family members, which directly control mitochondrial membrane permeability (154). The BCL-2 family of proteins can be divided into three subfamilies (3): the anti-apoptotic subfamily (Bcl-2, Bcl-w, myeloid cell leukemia differentiation protein [Mcl]-1 and Bcl-x_L), the multi-domain pro-apoptotic subfamily [Bcl-2 associated protein (Bax), Bcl-1 antagonist/killer (Bak), Mcl-2 and matador (Mtd)] and the still growing BCL-2 homology domain 3 (BH3)-only protein subfamily [Bcl-2/Bcl-XL associated death promoter (Bad), BH-3 interacting DD protein (Bid) and Bcl-2-interacting mediator of cell death (Bim)] which seem to function as death signal sensors.

The mitochondrial pathway can respond to both external and internal stresses. This suggests cross talk exists between the extrinsic and intrinsic pathways. For example, activated caspase-8 can also cleave Bid, resulting in the formation of truncated Bid (tBid), which can translocate to the mitochondria and activate the intrinsic pathway (134). Once the intrinsic pathway is activated, pro-apoptotic Bak and Bax increase the permeability of the mitochondrial membrane and release cytochrome c and apoptosis-inducing factor (AIF) into the cytoplasm. Release of cytochrome c results in its binding to apoptotic protease activating factor 1 (APAF-1), which, along with ATP, recruit and activate "initiator" caspase 9, forming a macromolecular complex called the apoptosome (197). Once the apoptosome is formed, caspase 9 activates effector caspases-3,-6 and -7 and apoptosis continues with death of the cell.

Activation of caspase-3, -6 and -7 results in the disassembly of the cell by cleaving a variety of vital cell proteins including those involved in DNA repair, nuclear lamins, and cytoskeletal proteins as well as activating the caspase-activated deoxyribonuclease which cuts genomic DNA into ~200 base pair fragments, all of which eventually culminate in the death of the cell (197).

Apoptosis as a Mediator of Trophoblast Differentiation

Apoptosis plays an important role in the regulation of trophoblast growth and turnover in the villous placenta. Morphological features of apoptosis have been identified in the villous placenta during normal development, indicating apoptosis is a necessary mediator for placental development (197). In addition to the removal of unnecessary cells, apoptosis has also been shown to play a role in cellular differentiation including

lens epithelial cell differentiation (which requires the activity of caspase 9) (182), leukocyte differentiation (which is mediated by caspase 8) (60), and monocyte maturation (which requires caspase 8) (184). When minor morphological changes are required, initiator caspases can be activated and when major structural changes are needed, effector caspases are stimulated (92).

Recent work by several groups has shown the apoptosis cascade, specifically the caspases, is involved in trophoblast differentiation. The premise behind this involvement is that phosphatidylserine externalization, an event that occurs early in the apoptotic cascade, is required for CT fusion and subsequently syncytialization and trophoblast differentiation. Work by Huppertz and colleagues have shown that areas of the ST expressing anti-apoptotic proteins Bcl-2 and Mcl-1 have intact nuclei whereas other regions, especially those located close to syncytial sprouts, have little or no Bcl-2 or Mcl-1 expression, accompanied by loss of nuclear proteins, the presence of PS externalization and TUNEL positive nuclei (88). Using isolated primary trophoblast cultures, Yusuf *et al* showed that cytotrophoblasts express higher caspase activity (specifically caspase-3, -6, -8,and -9) than ST and upon addition of EGF, a known stimulator of trophoblast differentiation, caspase activity was further reduced (215). This implies that the ST is less sensitive to apoptotic stimuli from external sources than the CT. More recently, Black and others have shown a requirement for caspase-8 activity in the differentiation and syncytialization of CT using a placental explant model. There are many problems with the conclusions drawn from this publication, given the lack of appropriate controls, lack of easily quantifiable data, use of a multi-cell explant to look at output from one cell type (in which caspase inhibition may effect more than one cell type involved in trophoblast differentiation), and the culture of explants at 6% oxygen which may leave the center of the explant in an hypoxic state (16). The authors used very low concentrations of inhibitors of caspase 8 and expression of caspase 8 in Western blots was normalized to vimentin, a marker which is not expressed by trophoblasts. Although phosphatidylserine externalization does occur during trophoblast differentiation, it was not inhibited by the broad spectrum caspase inhibitor ZVAD (53) implying that apoptosis and differentiation, although culminating in some of the same morphologic features, may in fact be mediated by distinct and unrelated signaling pathways.

Although *in vitro* evidence of caspase involvement in trophoblast differentiation remains to be convincingly demonstrated, apoptosis itself is evident in the villous placenta throughout pregnancy. In normal developing placenta, the morphological features of late apoptosis are never shown in the CT, as long as the overlying ST is intact (161). Upon CT fusion with the overlying ST, there is increased translation of anti-apoptotic proteins Bcl-2 and Mcl-1 (90). Structural signs of caspase 8 cleavage activity include cleavage of alpha-fodrin, a protein of the actin cytoskeleton, and externalization of phosphatidylserine within the ST (87, 89).

The progression of apoptosis does not occur immediately upon fusion of the CT with the ST (88). It is believed that the apoptotic cascade may be initially halted by the large amount of anti-apoptotic Bcl-2 family members present upon fusion (129, 181). The shift from inhibition of apoptosis to execution remains unclear; however, effector caspases 3 and 6 are eventually activated in the ST layer as demonstrated by caspase dependent cleavage of proteins such as CAD/ICAD, a DNase leading to the specific degradation of DNA (91), and cytokeratin 18, a major component of the cytoskeleton (108). The activity of effector caspases is temporally and spatially regulated and although the ST layer is a single multinucleated cell and does not have lateral membranes, DNA degradation and cytokeratin 18 cleavage are restricted to certain sites (91, 108). The last phase of apoptosis in the ST involves the accumulation of apoptotic nuclei into protrusions called syncytial knots. These knots are eventually shed from the ST into the maternal circulation (88, 149).

1.11 Abnormal Placentation

Abnormal placentation is a common feature among both IUGR and preeclampsia. Although both diseases are associated with placental insufficiency, it remains unclear if placental development is the main cause of these conditions or if other factors are involved.

1.11.a Intrauterine Growth Restriction

Intrauterine growth restriction (IUGR) is a failure of a fetus to reach its optimal growth potential (116). IUGR fetuses have increased risk of perinatal mortality, and following birth an increased risk of cognitive dysfunction and cerebral palsy. As well, epidemiological studies have shown that the in-utero environment of IUGR predisposes individuals for coronary heart disease, hypertension, stroke and diabetes during adulthood (9, 10). IUGR is associated with maternal smoking, infection, maternal illness and chromosomal abnormalities, but can also be associated with idiopathic placental failure in which placental development is abnormal and suboptimal levels of nutrients and oxygen are supplied to the fetus (178).

IUGR implies that fetal growth is limited by one or more underlying pathological processes. In contrast, "small for gestational age [SGA]" implies appropriate fetal growth when accounting for parental size and ethnicity and is usually associated with normal placental function (34). As such, IUGR and SGA are often hard to distinguish, especially in the late third trimester of pregnancy. "Early-onset" IUGR, defined as disease requiring delivery before 32 weeks gestation, may be due to a number of factors including congenital infection [such as human cytomegalovirus], aneuploidy, a range of genetic disorders and uteroplacental vascular insufficiency, characterized by abnormal Doppler studies in the uterine and umbilical arteries (34). Early-onset IUGR is much more serious for the developing fetus than late-onset IUGR and is characterized by reduced, absent or reversed end-diastolic flow velocity in the umbilical arteries in addition to abnormalities in uterine artery Doppler waveforms (34, 201). In contrast, late-onset IUGR is typically associated with asymmetric fetal growth, normal umbilical and uterine artery Doppler flow, low amniotic fluid and a mature placenta on ultrasound (82). Part of the complications surrounding the study of IUGR is the lack of a consistent method for identification in the literature. In the majority of studies, IUGR babies are those born less than the 10^{th} percentile for their gestational age, which could exclude those babies which weighed more than the 10^{th} percentile but were still growth restricted *in utero* (205).

1.11.b Preeclampsia

Preeclampsia is a pregnancy associated syndrome affecting mainly the maternal system and is characterized by maternal hypertension (140/90 mmHg) and proteinuria (>0.3 g/24 hours or >2+ on protein strip) (203). Preeclampsia is associated with IUGR in approximately one-third of cases (62). Preeclampsia, like IUGR, is also responsible for significant perinatal mortality and morbidity as a result of premature delivery, which is performed to reverse the clinical course of the disease and accounts for approximately 15 percent of all premature births (177).

1.11.c Characteristic Features of Placentae from Preeclamptic/IUGR pregnancies

There is increasing evidence that perturbations of placental development can lead to compromised fetal outcome, such as intrauterine growth restriction (IUGR) and preeclampsia (13). Indeed, shallow trophoblast invasion leading to insufficient implantation is a key pathologic feature of both IUGR and preeclamptic pregnancies (23). During the first half of pregnancy, uteroplacental arteries undergo a number of changes including replacement of endometrium and smooth muscle cells by invasive extravillous trophoblast and loss of elasticity and vasomotor control (24). These changes result in the formation of wide incontractile tubes establishing a low resistance blood supply to the developing fetus. Subsequently, inadequate trophoblast invasion leads to reduced uteroplacental artery remodeling, impairing oxygen and nutrient delivery to the placenta and fetus.

There are several characteristic pathologic developmental features of IUGR and pre-eclamptic placentae which contribute to reduced uterine artery blood flow and consequently a decreased supply to the developing fetus (34). These include: reduced extravillous trophoblast invasion, leading to poor transformation of the uterine spiral arteries resulting in high uteroplacental resistance (34); decreased lumen diameter of umbilical artery, which impedes flow and leads to high resistance; abnormal development of mature intermediate villi within the placenta, characterized by reduced amounts of proliferating villous CT overlaid by an aged apoptotic ST layer (89); and elevated levels of thrombo-occlusive lesions within the placenta, reducing placental blood flow (79).

As discussed previously, apoptosis is an important process in the development of the human placenta, but elevated levels of apoptosis in the villous trophoblast have been demonstrated in IUGR (61, 95, 190). Villous tissue revealed enhanced p53 tumor-suppressor protein (p53) expression which can activate proapoptotic Bcl-2 family members such as Bax (132). Increased levels of TNFα were found in the maternal serum (11) and amniotic fluid (194) from pregnancies complicated with IUGR. IUGR placentae exhibited elevated levels of TNFα production (85). In addition, cultured CT, ST and villous explants displayed increased susceptibility to the proapoptotic effects of TNFα (45, 46). These observations suggest that an inflammatory environment exists within the IUGR placenta, leading to apoptosis, and TNFα may be involved in the mechanisms underlying the pathophysiology of IUGR.

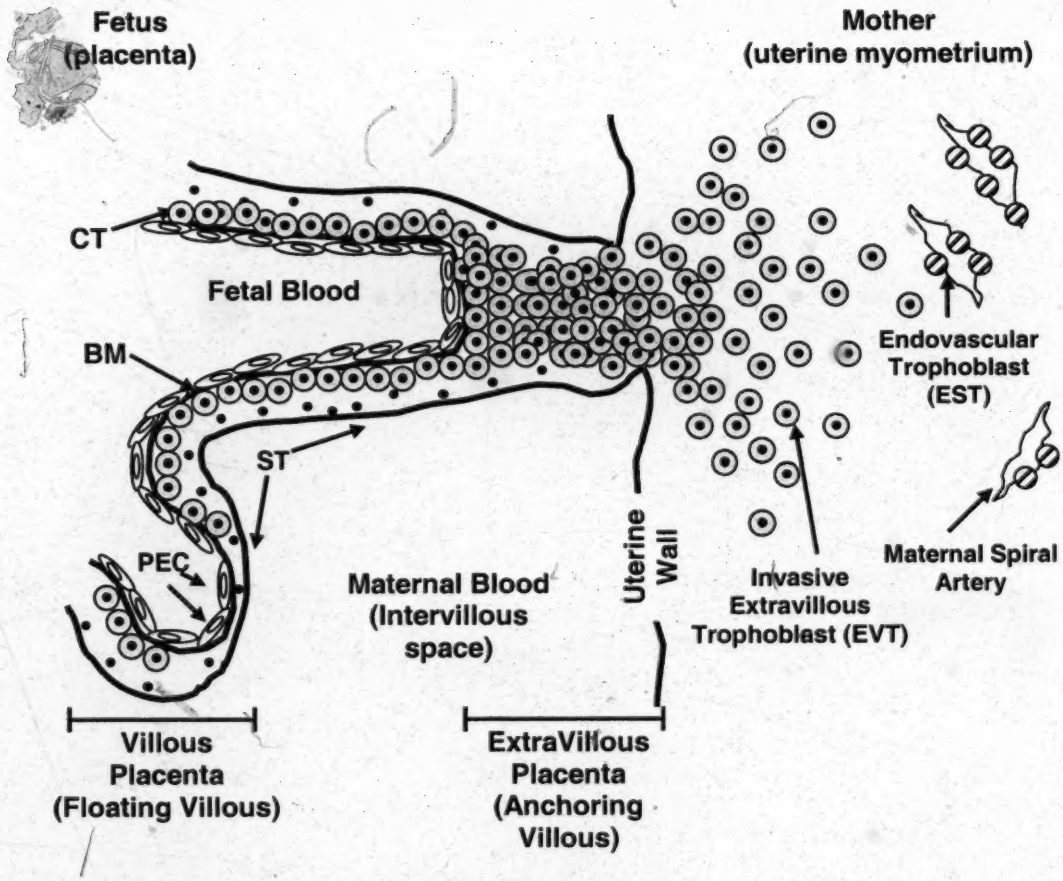


Figure 1.1: Diagram of the utero-placental interface

Extravillous trophoblasts (EVT) invade the maternal uterine wall to form the anchoring villous placenta, after which endovascular trophoblast (EST) invades the maternal spiral arteriers and connects fetal and maternal tissue. The floating villous placenta is bathed in maternal blood and mediates the exchange of nutrients and oxygen between the mother and developing fetus. As the floating villous branches, areas of syncytiotrophoblast (ST) come in direct contact with placental endothelial cells (PEC), allowing for optimal exchange and oxygen uptake into the fetal circulation. The basement membrane (BM) is composed of extracellular matrix molecules.

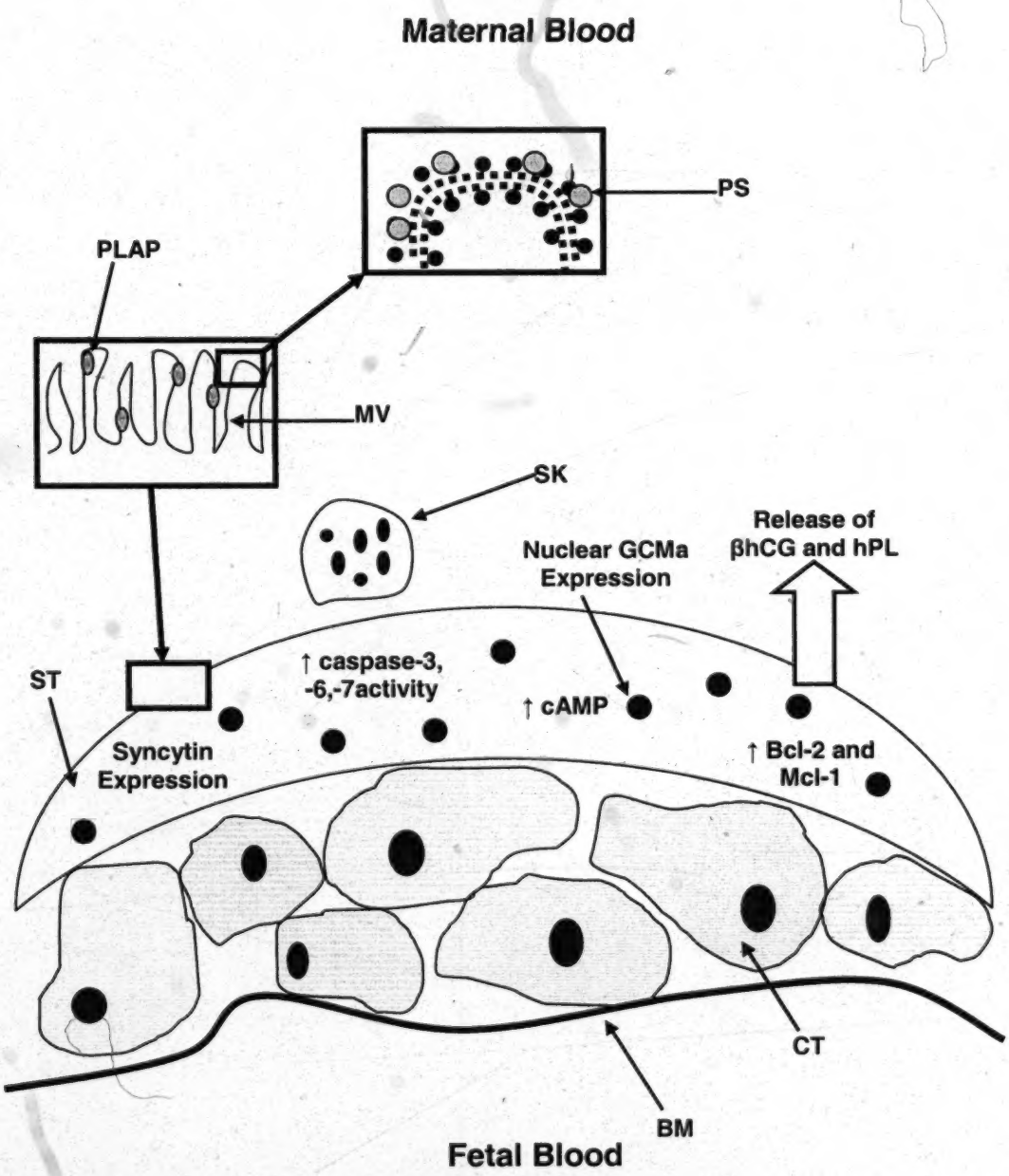


Figure 1.2: Characteristic features of differentiated syncytiotrophoblast.

Differentiated syncytiotrophoblast (ST) form microvillus structures to increase surface area, upon which placental alkaline phosphatase (PLAP) is expressed. Along the surface of the ST, phosphatidylserine (PS) is externalized, a process involved in the differentiation of cytotrophoblast (CT) to ST. ST express high levels of anti-apoptotic Bcl-2 and Mcl-1 proteins, high levels of syncytin (or HERV-W), elevated intracellular cAMP levels, and nuclear localization of the transcription factor GCMa.

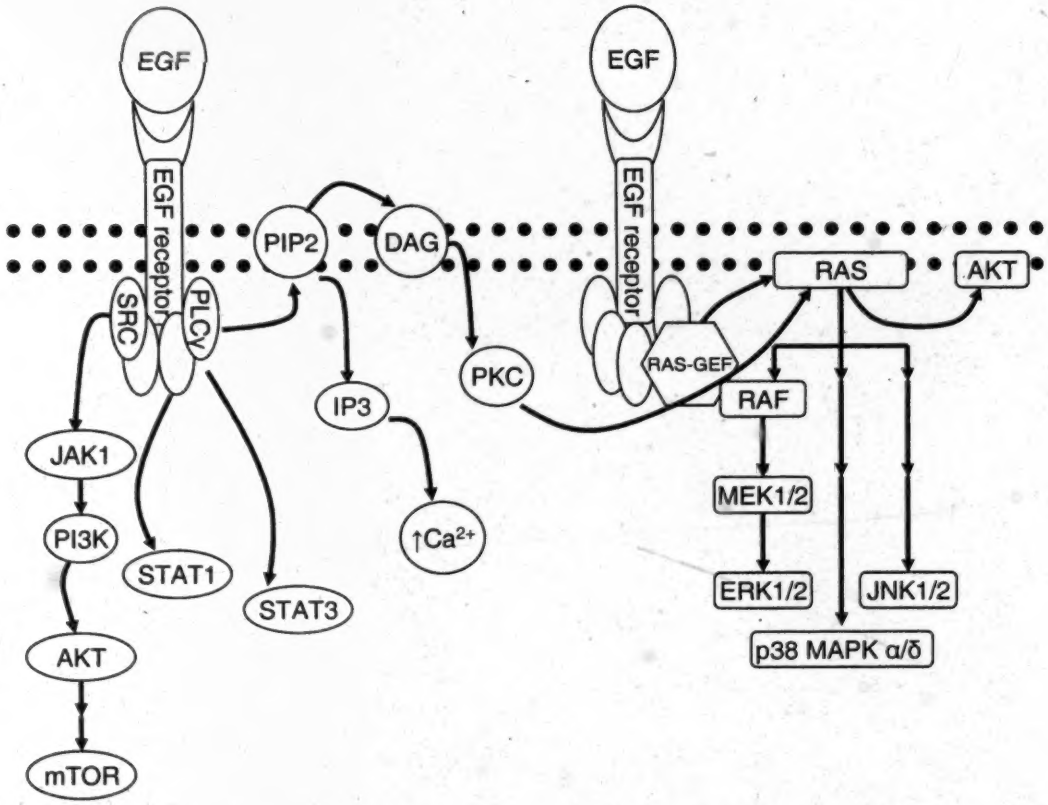
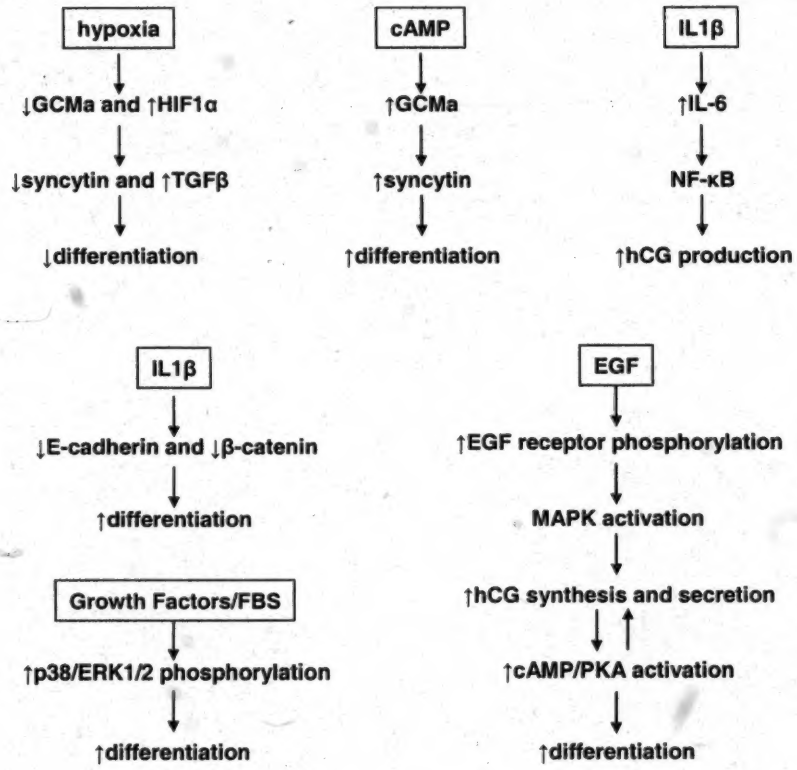


Figure 1.3: Signaling pathways activated by epidermal growth factor (EGF) in trophoblasts.

EGF binding to EGF receptors results in EGF receptor dimerization, autophosphorylation of the receptor and tyrosine phosphorylation of other proteins. The EGF receptor activates Ras (a guanine nucleotide binding protein) which in turn recruits Raf which is the main effector of MEK-MAPK pathway activation, ultimately resulting in the phosphorylation of transcription factors such as c-FOS, c-JUN, AP-1, and ELK-1 which contribute to proliferation, differentiation, and survival signaling. In addition to activation of MAPK cascade, Ras activation also results in the activation Akt (also known as protein kinase B or Rac) which stimulates cell survival signaling and cell cycle regulation. SRC family kinase signaling leads to the activation of STAT-1 and STAT-3 transcription factors by Janus family tyrosine kinases [JAK] in response to EGF contributes to proliferative signaling as well as the activation of phosphoinositide 3-kinase [PI3K]. Phosphatidylinositol signaling through phosphoinositide-specific phospholipase C gamma [PLCγ] stimulation of phosphatidylinositol-4,5-bisphosphate [PIP2] and diacylglycerol [DAG] results in the activation of protein kinase C [PKC] and [IP3] and calcium release from the endoplasmic reticulum induced by EGF. mTOR (mammalian target of rapamycin) acts as sensor for ATP and amino acids and plays a key role in cell growth and homeostasis. Adapted from www.biocarta.com.



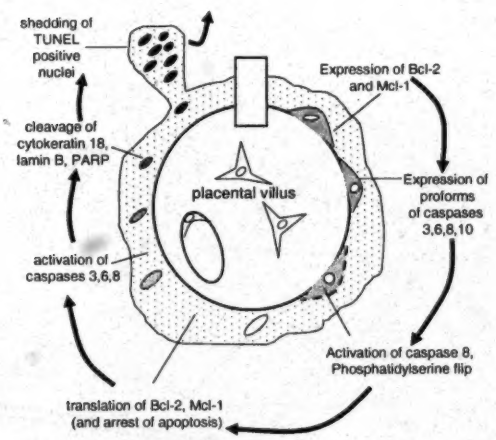

shedding of TUNEL positive nuclei
cleavage of cytokeratin 18, lamin B, PARP
activation of caspases 3,6,8
translation of Bcl-2, Mcl-1 (and arrest of apoptosis)
Activation of caspase 8, Phosphatidylserine flip
Expression of proforms of caspases 3,6,8,10
Expression of Bcl-2 and Mcl-1
placental villus

Figure 1.4: Major signaling pathways involved in trophoblast differentiation.

Multiple signaling pathways are involved in trophoblast differentiation. For simplicity, individual pathways are separated, although they may interact with each other within the trophoblast. The bottom figure was adapted from Huppertz *et al* (93).

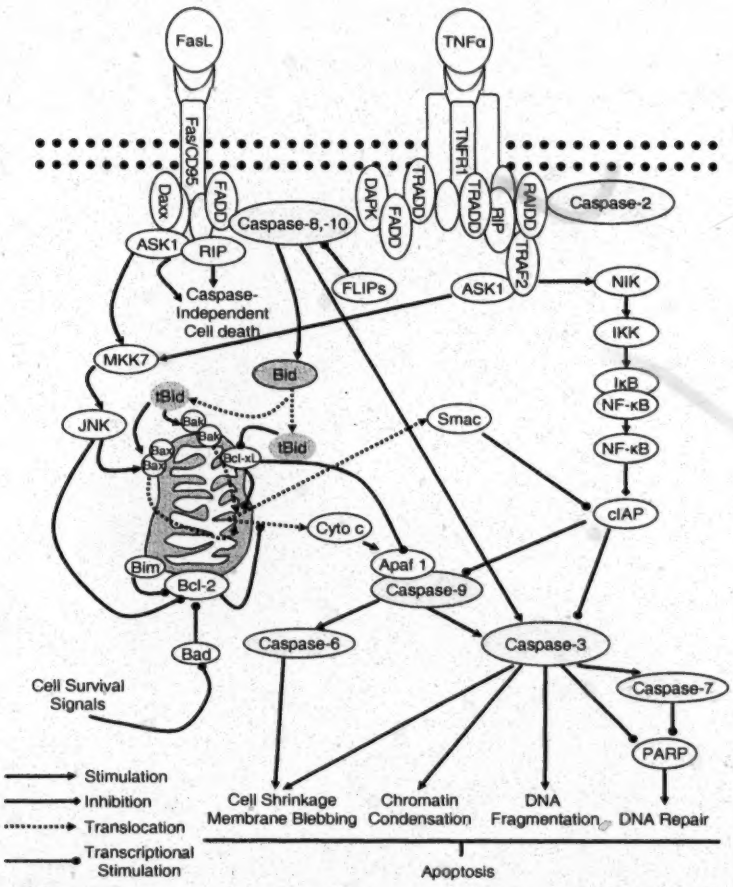


Figure 1.5: Apoptosis cascade: extrinsic and intrinsic pathways of apoptosis.

The extrinsic pathway is mediated by external ligand (either TNFα or FasL) binding to receptors such as TNF receptor 1 and Fas/CD95 found on trophoblasts. Death receptor signaling results in oligomerization of receptors and recruitment of adaptor proteins (FADD, TRADD, Daxx, ASK1, RIP, DAPK) and activation of initiator caspase 8. Activation of caspase-8 results in activation of the caspase cascade directly through activation of caspase-3 and indirectly through cleavage of Bid and activation of the intrinsic pathway. Alternatively or simultaneously TNFα binding to TNFR can activate the NF-κB pathway via an adaptor protein complex including RIP and survival genes including IAP. The intrinsic pathway is controlled by the Bcl-2 family of proteins. Anti-apoptotic Bcl-2 and Bcl-xL remain on the outer mitochondrial wall and inhibit cytochrome c release. Proapoptotic Bcl-2 family proteins Bad, Bak, Bax and Bim remain in the cytosol and translocate to the mitochondria upon death signaling where they act to promote the release of cytochrome c. The intrinsic pathway is also mediated by translocation of truncated Bid which induces the release of cytochrome c from the mitochondria, where it binds to Apaf1 and forms the apoptosome complex with caspase 9 resulting in the activation of effector caspases-3, -6, and -7. Adapted from www.cellsignal.com.

CHAPTER 2: RATIONALE AND HYPOTHESIS

2.1 Hypothesis

IUGR is a serious pregnancy complication in which the fetus fails to reach its full optimal growth potential and the etiology is unknown. IUGR has been associated with a number of potential causes including maternal smoking, maternal undernutrition and placental insufficiency and occurs in approximately 8 to 10% of all pregnancies (202). In addition to increasing perinatal mortality and morbidity, IUGR is also associated with increased prevalence of adult disease such as cardiovascular problems and diabetes (81). The likelihood of placental insufficiency as the main cause of IUGR (81) makes understanding placental development in IUGR of the utmost importance.

Placental development is a tightly controlled and highly complex process involving the coordinate regulation of multiple cell types. The extravillous trophoblast is responsible for mediating the transformation of the maternal spiral arteries required for adequate blood flow to the developing fetus where as the syncytiotrophoblast, a multinucleated syncytium which covers the highly branched villous trees, is responsible for secreting hormones to maintain a successful pregnancy in addition to maintaining a functional barrier between the maternal and fetal circulatory systems. The ST is also responsible for nutrient and oxygen uptake from the maternal blood and is accountable for adequate nutrient supply to the developing fetus. The ST layer is maintained by fusion of underlying proliferative CT which serves as stem cells to maintain the syncytium.

Apoptosis is a key regulator of differentiation in multiple cell types and the presence of apoptotic nuclei in normal placenta indicate that apoptosis is an important modulator of placental development. However, in IUGR placentae there are excess numbers of apoptotic nuclei and poor development of the highly branched villous structures, suggesting that excess apoptosis may decrease the number of CT available for differentiation and subsequently contribute to the pathophysiology of IUGR placenta. IUGR is also associated with increased TNFα production, implicating a role for TNFα in

this disease. **My hypothesis is that increased TNFα production in IUGR placentae contributes to increased rates of trophoblast apoptosis and decreased trophoblast differentiation both of which contribute to decreased villous placental function leading to compromised fetal growth.**

2.2 Rationale

I chose to study trophoblast differentiation in IUGR and normal trophoblasts for the following reasons:

1. IUGR is a very serious pregnancy complication affecting approximately 8-10% of all live births. IUGR is also associated with increased perinatal morbidity and mortality as well adult health complications including cardiovascular disease and diabetes. Although IUGR has been studied for many years, the etiology of idiopathic IUGR remains unknown. IUGR is often associated with preeclampsia, a maternal disease associated with 2-6 % of pregnancies.

2. IUGR and preeclamptic placentae are both associated with incomplete placentation and poor extravillous trophoblast invasion of maternal spiral arteries, implicating a role for the placenta in these diseases. Inadequate trophoblast invasion results in decreased blood supply to the developing fetus which has a negative impact on fetal growth.

3. IUGR and preeclamptic placentae have been shown to produce higher levels of the pro-inflammatory cytokine TNFα. IUGR and preeclamptic placentae also have elevated levels of apoptosis in both the syncytiotrophoblast and cytotrophoblast. In addition, cells from IUGR and preeclamptic placentae have shown increased sensitivity to the effects of TNFα, resulting in increased apoptosis.

4. Our lab has developed a technique to isolate highly purified human primary trophoblast (>99.99% pure) from normal placenta. This technique can also be applied to IUGR and preeclamptic placentae. Normal villous CT can be cultured for periods of up to two weeks with the addition of EGF and undergo both spontaneous and EGF-induced differentiation under standard culture conditions. These cultures have been utilized to

study trophoblast apoptosis in IUGR and normal placentae but little is known about how IUGR trophoblasts differentiate in culture.

2.3 Experimental Design

My initial experiments were to determine if differentiation (assessed by hCG production [ELISA], multinucleation [desmoplakin staining], and placental alkaline phosphotase staining) was different between populations of normal and IUGR trophoblast in culture under standard conditions. This study showed that the differentiation markers hCG and multinucleation were significantly higher in IUGR trophoblasts than in normal and that IUGR-PE trophoblasts behaved similarly to normal in culture (Chapter 3). As IUGR-PE behaved differently to IUGR, I wanted to establish why IUGR was different from normal. In addition to increased differentiation, there was also increased apoptosis, as assessed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining, in IUGR trophoblasts.

My second objective was to determine if TNFα played a role in both the increased apoptosis and differentiation seen in IUGR. As TNFα is known to be increased in IUGR and TNFα mediates enhanced trophoblast apoptosis (Kilani *et al*, in preparation), neutralizing antibody to TNFα was added to cultures and was able to decrease both apoptosis and syncytialization in IUGR trophoblasts to levels seen in normal trophoblast cultures (Chapter 3). This suggested a role for TNFα in trophoblast differentiation. TNFα has been shown in other epithelial cells to activate the phosphorylation of p38 MAPK, ERK1/2 [both of which have been shown to play a role in trophoblast differentiation], and JNK [which is involved in trophoblast survival signaling] MAPKs as well as the cleavage of caspases and the apoptotic cascade. When cytotrophoblast cultures were stimulated with TNFα, there was activation of p38, ERK1/2 and JNK (Chapter 3) as well as caspase cleavage.

My third objective was to establish which pathway TNFα was signaling through to stimulate increased differentiation in IUGR trophoblasts. Using pharmacologic inhibitors to p38 MAPK (SB203580), ERK1/2 (PD098590), JNK (JNK II Inhibitor), and caspases (ZVAD), I found that inhibition of ERK1/2 activity prevented the elevated level

of multinucleation seen in IUGR trophoblasts (Chapter 3). Inhibition of SB203580 also decreased both functional (hCG) and morphologic differentiation in IUGR. JNK inhibition did not effect trophoblast differentiation. Caspase inhibition had no effect on trophoblast differentiation in IUGR or normal trophoblasts, indicating that the caspase cascade is not involved in trophoblast differentiation in isolated cells *in vitro*.

My final aim was to confirm that TNFα could stimulate trophoblast differentiation. When exogenous TNFα was added to normal trophoblasts, there was an elevation in multinucleation but a decrease in hCG production in a dose dependent manner (Chapter 3). Addition of both TNFα and the ERK1/2 inhibitor PD098590 prevented TNFα stimulated multinucleation but did not decrease hCG production (Chapter 3). TNFα was shown to act through both ERK1/2 and p38 MAPK to stimulate multinucleation and hCG production respectively.

CHAPTER 3: MATERIALS AND METHODS

3.1 Patient Population

IUGR fetuses were identified before birth at the Royal Alexandra Hospital Women's Center in Edmonton, Alberta on the basis of ultrasound information indicating compromised fetal growth. The criteria for IUGR include: estimated birthweight less than the 10th centile for gestational age accompanied by either: 1) head/abdominal circumference ratio less than 1.2, 2) low amniotic fluid level (less than 7 cm), or 3) abnormal umbilical Doppler flow. Birthweights less than the 10th centile were confirmed after birth. IUGR fetuses born to pre-eclamptic mothers were further characterized by maternal hypertension (blood pressure of 140/90 mmHg) and proteinuria (greater than +2) during the latter half of pregnancy. Normal pregnancies were those babies born between the 10th and 90th centile without other complications.

3.2 Villous Cytotrophoblast Isolation

Villous cytotrophoblast cells were isolated following the protocol developed by Morrish *et al* (1987). Briefly, freshly delivered placentae were trimmed of membranes, cut into cotyledons and thoroughly rinsed in IMDM/2% calf serum. Tissue was scraped off of underlying vasculature and rinsed again with IMDM/2% calf serum through a metal sieve [40 mesh-Sigma]. Tissue was incubated with digestion solution (2.5 μg/ml trypsin [Gibco 1:250 trypsin #27250-018], 0.1 μg/ml DNAse [Sigma] in Locke Ringer's [0.9% NaCl, 0.042% KCl, 0.01-0.03% $NaHCO_3$, 0.1-0.2% glucose] buffer) at a ratio of 1 g tissue to 1 mL solution at 37°C. The first two rounds of trypsinized cells were discarded, with 5 subsequent rounds of trypsinized cells being collected and pooled in cold PBS containing 2% calf serum [PBS/2%CS] (GibcoBRL) on ice. Following trypsinization, cells were further purified according to methods established by Yui *et al* (212) and Guilbert *et al* (75), red blood cells were lysed by incubation with lysis buffer [150 mM ammonium chloride, 10 mM sodium bicarbonate, 0.1 mM disodium EDTA] for 8 minutes at room temperature. Cells were then pelleted and washed with cold

PBS/2%CS twice and then incubated with 10% (v/v) normal goat serum in 3 ml cold PBS/2%CS for 20 minutes on ice to block non-specific antibody interactions. Cells were then suspended in an additional 40 ml PBS/2% CS, pelleted and incubated with 3 ml of primary antibody solution (PBS/2%CS with mouse anti-human MHC-I, MHC-II and CD9 monoclonal antibodies) for 45 minutes on ice. The cells were pelleted in 50 ml of PBS/2%CS and resuspended in cold PBS/2%CS at a concentration of $\leq 100 \times 10^6$ cells/5 mL. The cell suspension (~100 x 10^6 cells/column) was then loaded onto goat-antimouse IgG coated glass bead columns and run through with cold PBS/2%CS to maintain constant flow. Cells which passed through the columns (therefore negatively selected) were collected, pelleted, and resuspended in cold calf serum with 10% DMSO (v/v) to produce 1 x 10^7 cells/mL. Cells were aliquoted into freezing vials and frozen at -70°C overnight and were then transferred to LiN_2 for long term storage.

3.3 Villous Cytotrophoblast Culture

Thawing of Cryopreserved Stocks and Basic Culture

Trophoblasts were thawed at 37°C after cryopreservation into 10 mL of IMDM/2% FCS and centrifuged at 1500 rpm for 7 minutes in a Beckmann CS-6R centrifuge at 4°C. Cells were resuspended in IMDM/10% FCS/2%Pen/Strep stock [10,000 U/ml penicillin and 10,000 μg/ml streptomycin]/1%L-glutamine at a density of 1×10^6 cells per mL of media. Cells were plated in 96 well NUNC plates at 150 μL/well (1.5×10^4 cells per well) and allowed to adhere for a minimum of 4 hours in a 37°C ambient air/5% CO_2 incubator. After 4 hours, non-adherent cells and debris were removed by gentle washing with IMDM/2%FCS, after which all media was removed and replaced with fresh IMDM/10%FCS/2%Pen/Strep/ 1%L-glutamine [IMDM-complete]. Cells were cultured for 5 days, media being changed on day 1 and then every other day. Concentrations of each treatment are listed in detail in **Table 3.1** below.

Culture with Peptide Inhibitors

Trophoblasts were routinely thawed and plated for 4-6 hours in 96 well plates at the density mentioned above, after which the cultures were washed as described above

and media was replaced with IMDM-complete media in which inhibitors were diluted. All peptide inhibitors were dissolved originally in DMSO, final inhibitor concentrations being 10 μM [SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole), PD098590 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), SB202474 (4-Ethyl-2(p-methoxyphenyl)-5-(4′-pyridyl)-IH-imidazole), SB202190 (4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol)], 20 μM [ZVAD-fmk], or 90 nM [JNK II Inhibitor and JNK II negative control inhibitor]. Upon dilution, DMSO concentration was between 0.1 and 0.3%, concentrations of which were added to separate wells as vehicle treatment. **Figure 3.1** shows where the inhibitors act in the TNFα signaling cascade.

Culture with Neutralizing Antibodies

Trophoblasts were routinely thawed and plated at a density of $1.5x10^4$ cells per well in 100 μL of media in 96 well plates, 50 μL of media containing 7.5 μg/ml of neutralizing antibody to TNFα, IL1β or IgG_1 isotype control were added to plated cells within 5 minutes of plating. Cells were allowed to adhere for 4-6 hours and then washed with 2%FCS/IMDM. Media was replaced with fresh media containing antibodies added to a final concentration of 2 (IL-1β) or 2.5 (TNFα) μg/ml in IMDM-complete. 2 μg/ml of neutralizing antibody to IL-1β should neutralize 50 ng/mL of IL-1β and 2.5 μg/ml of neutralizing antibody to TNFα should neutralize of 20 ng/mL TNFα.

Culture with TNFα, EGF and 8-bromo-cAMP

Trophoblasts were routinely thawed and plated at the density of $1.5x10^4$ cells per well in 150 μL of media in 96 well plates. Cells were allowed to adhere for 4-6 hours, after which non-adherent cells were washed with 2%FCS/IMDM. Media was replaced with fresh media containing concentrations of TNFα (0.01, 0.1, 1 and 10 ng/ml), EGF (10 ng/ml), or 8-bromo-cAMP (10^{-5} M) diluted in IMDM-complete.

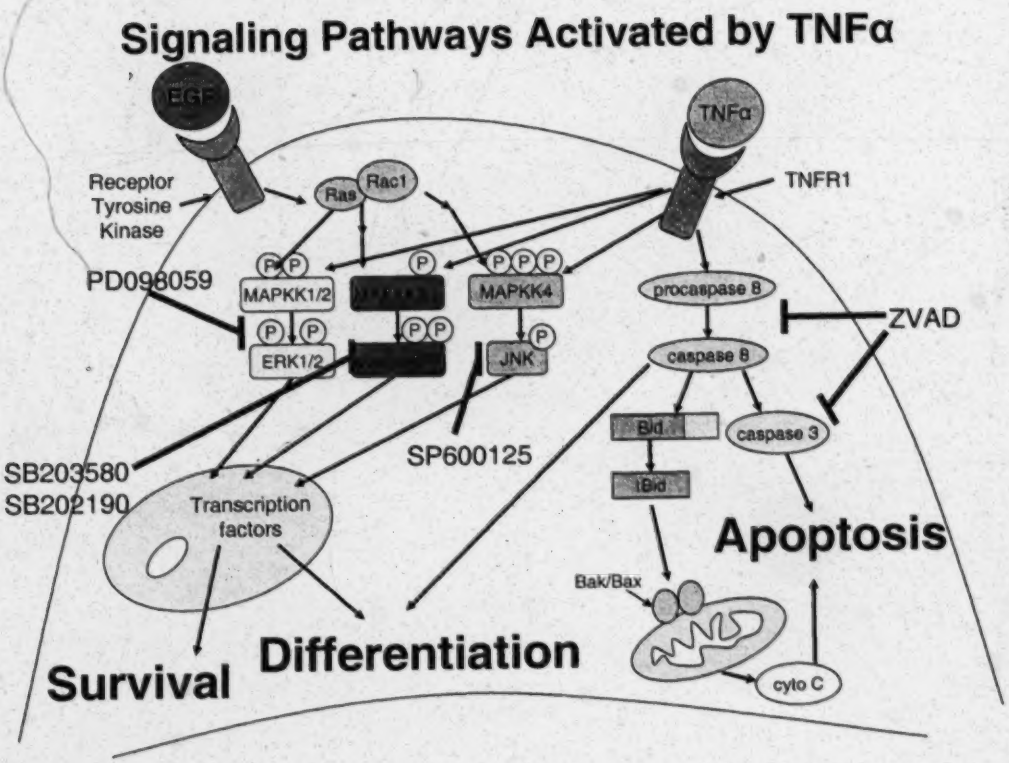


Figure 3.1 Site of action of peptide inhibitors of p38 MAPK, ERK1/2 and JNK MAPK and the caspase inhibitor ZVAD-fmk.

Table 3.1. Culture treatments and concentrations.

Treatment	Final Concentration	Time Added	Company
Peptide Inhibitors			
DMSO Vehicle	0.1 to 0.3%	After washing	Sigma
p38 MAPKα/β inhibitor SB203580	10 μM	After washing	Calbiochem
p38 MAPKα/β inhibitor SB202190	10 μM	After washing	Calbiochem
negative control for p38 MAPKα/β inhibitors SB202474	10 μM	After washing	Calbiochem
MEK1/2 (ERK1/2 inhibitor) PD098590	10 μM	After washing	Sigma
JNK II Inhibitor SP600125	90 nM, 10 μM	After washing	Calbiochem
JNK II Inhibitor negative control	90 nM, 10 μM	After washing	Calbiochem
ZVAD	20 μM	After washing	R & D Systems
Antibodies			
IgG_1 isotype control	2.5 ug/mL	At plating	Dakocytomation
Neutralizing antibody to hTNFα (IgG_1) clone 2C8	2.5 μg/ml (ND_{50} is 0.3 ng/ml)	At plating	Upstate Biotechnology, Lake Placid, NY
Neutralizing antibody to hIL-1β (IgG_1)	2μg/ml (ND_{50} of 50 pg/ml is 0.001μg/ml)	At plating	R&D Systems, Minneapolis, MN
Growth, Differentiation and Death Inducing Factors			
Recombinant human TNFα	0.01, 0.1, 1, and 10 ng/ml	At plating or After washing	Hoffman-LaRoche
recombinant human EGF	10 ng/ml	After washing	Pepro-Tech, Rocky Hill, NJ
8-bromo-cAMP	10^{-5} M	After washing	Sigma

Culture at 5% and 2% Oxygen

Trophoblasts were routinely thawed and plated at a density of $1.5x10^4$ cells per well in 150 μL of media. Cells were allowed to adhere for 4-6 hours at one of three oxygen tensions (all environments were fully humidified): 1) ~140 mmHg or in a standard 5 per cent CO2 in air incubator; 2) ~38 mmHg or in a controlled oxygen

incubator (Forma Series II, Forma, Marietta, OH, USA) regulated at 7 per cent CO_2 and at 5 per cent oxygen; or 3) ~ 15 mmHg or in a modular-incubator chamber (Billups-Rothenberg, DeMar, CA, USA) in which air was replaced with nitrogen containing 2 percent oxygen and 5 per cent CO_2 by a time and flow (15.0 min, 200 mL/sec) controlled purge, after which non-adherent cells were washed with 2%FCS/IMDM. Media was replaced with 100 μL fresh media [IMDM-complete] and plates were returned to respective oxygen tensions. Media was pre-warmed and changed at 24 and 72 hours, plates being out of the incubator for less than 5 minutes.

3.4 Preparation of Cell Lysates for Western Blot Analysis

Plating of Cells For Western Blot Analysis

Trophoblasts were thawed into IMDM/2%FCS, centrifuged and resuspended in IMDM-complete media at a density of 3.33×10^6 cells per mL. Cells were plated at a density of 3.33×10^6 cells per well in Costar 6-well tissue culture plates and allowed to adhere for 6 hours in a 37°C ambient air/5% CO_2 incubator. Non-adherent cells and debris were removed by gentle rocking of the plate and rinsing with IMDM/2%FCS. Media was replaced with IMDM-complete.

Phosphorylation of p38 MAPK in Cytotrophoblasts

Normal trophoblasts were cultured as described above for 18 hours in an ambient air/5% CO2 incubator. Media was removed and inhibitors SB203580 or SB202190 were added at a final concentration of 10 μM and cells were returned to the incubator for 1 hour. For EGF and TNFα stimulation of p38 MAPK phosphorylation, cytokines were added to a final concentration of 10 ng/ml with or without inhibitors SB203580 or SB202190 in IMDM-complete media with 3 wells per treatment. At time points of 5, 10, 15, 20 and 30 minutes after addition of EGF or TNFα, media was removed, cells were rinsed with cold PBS and placed on ice. PBS was removed and 100 μL of 2X SDS-PAGE loading buffer [100 mM Tris-Cl (pH 6.8), 20% β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue and 10% glycerol] was added to each well. Cells were scraped and

lysates from 3 individual wells were pooled into one tube. Lysates were then boiled for 7 minutes and stored at -20°C until use.

Phosphorylation of ERK1/2 in Cytotrophoblasts

Normal trophoblasts were cultured as described above for 18 hours in an ambient air/5% CO2 incubator. Media was removed and the inhibitor PD098590 was added at a final concentration of 10 μM and cells were returned to the incubator for 1 hour. For EGF and TNFα stimulation of ERK1/2 phosphorylation, cytokines were added to a final concentration of 10 ng/ml with or without inhibitor PD098590 in IMDM-complete media with 3 wells per treatment. At time points of 5, 10, 15, 20 and 30 minutes after addition of EGF or TNFα, media was removed, cells were rinsed with cold PBS and placed on ice. PBS was removed and 100 μL of 2X SDS-PAGE loading buffer was added to each well after which the cells were scraped and lysates from 3 individual wells were pooled into one tube. Lysates were then boiled for 7 minutes and stored at -20°C until use.

Phosphorylation of JNK in Cytotrophoblasts

Normal trophoblasts were cultured as described above for 18 hours in an ambient air/5% CO2 incubator. Media was removed and the inhibitor JNK II Inhibitor was added at a final concentration of 10 μM and cells were returned to the incubator for 1 hour. For EGF and TNFα stimulation of JNK phosphorylation, cytokines were added to a final concentration of 10 ng/ml with or without JNK II Inhibitor in IMDM-complete media with 3 wells per treatment. At 30 minutes after addition of EGF or TNFα, media was removed, cells were rinsed with cold PBS and placed on ice. PBS was removed and 100 μL of 2X SDS-PAGE loading buffer was added to each well. Cells were scraped and lysates from 3 individual wells were pooled into one tube. Lysates were then boiled for 7 minutes and stored at -20°C until use.

Syncytin and GCM-1 Expression in Mixed Cytotrophoblast/Syncytiotrophoblast Culture

Normal and IUGR trophoblasts were thawed as described above and after washing were cultured in either IMDM-complete, IMDM-complete supplemented with 10ng/ml EGF, or IMDM-complete supplemented with 10^{-5} M 8-bromo-cAMP for 3 days,

media being changed after 24 hours, in an ambient air/5% CO2 incubator. After 72 hours of culture, cells were rinsed with cold PBS and placed on ice. PBS was removed and 100 μL of lysis buffer [1% TritonX-100, 10% Glycerol, 2mM EDTA, 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM PMSF, 10% Phosphatase Inhibitor Cocktail (Sigma), 10% Mammalian Protease Inhibitor Cocktail (Sigma)] was added to each well. Cells were scraped and lysates from 3 individual wells were pooled into one tube. Lysates were incubated on ice for 15 minutes, after which they were frozen at -20°C. Protein concentration of total cell lysates was determined by the BCA method. 30 μg of total protein was mixed with SDS-PAGE loading buffer to a final concentration of 10% β-mercaptoethanol, 10% glycerol, 2% SDS, 0.1% Bromophenol blue, 50 mM Tris-HCl, pH 6.8 and boiled for 7 minutes. Insoluble matter was pelleted briefly by centrifugation.

Caspase-8 Cleavage in Cytotrophoblasts

Trophoblasts were routinely thawed and cultured in an ambient air/5% CO2 incubator for 4-6 hours in IMDM-complete, after which they were washed with IMDM/2% FCS and cultured in IMDM complete for 18 hours. The following day, the following treatments were applied as follows: control (IMDM-complete) for 12 hours, ZVAD (20 μM ZVAD in IMDM-complete) for 12 hours, TNFα (10 ng/ml in IMDM-complete) for 6 and 12 hours, TNFα + ZVAD (10 ng/ml TNFα and 20 μM ZVAD in IMDM-complete) for 6 and 12 hours. The cultures were returned to the incubator for the specified time periods, after which the media was removed and the cultures were rinsed with cold PBS and placed on ice. PBS was removed and 100 μL of lysis buffer [1% TritonX-100, 10% Glycerol, 2mM EDTA, 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM PMSF, 10% Phosphatase Inhibitor Cocktail (Sigma), 10% Protease Inhibitor Cocktail (Sigma)] was added to each well. Cells were scraped and lysates from 3 individual wells were pooled into one tube. Lysates were incubated on ice for 15 minutes, after which they were frozen at -20°C until use. Protein concentration of total cell lysates was determined by the BCA method. 40 μg of total protein was mixed with SDS-PAGE loading buffer to a final concentration of 10% β-mercaptoethanol, 10% glycerol, 2% SDS, 0.1% Bromophenol blue, 50 mM Tris-HCl, pH 6.8 and boiled for 7 minutes. Insoluble matter was pelleted briefly by centrifugation.

Caspase-3 Cleavage in Cytotrophoblasts

Trophoblasts were routinely thawed and cultured in an ambient air/5% CO2 incubator for 4-6 hours in IMDM-complete, after which they were washed with IMDM/2% FCS and cultured in IMDM complete for 18 hours. The following day, the following treatments were applied for 18 hours as follows: control (IMDM-complete), cycloheximide (10 μg/ml in IMDM-complete), TNFα (10 ng/ml in IMDM-complete), TNFα + ZVAD (10 ng/ml TNFα and 20 μM ZVAD in IMDM-complete), TNFα + cycloheximide (10 ng/ml TNFα and 10 μg/ml cycloheximide in IMDM-complete), and TNFα + ZVAD + cycloheximide (10 ng/ml TNFα, 10 μg/ml cycloheximide and 20 μM ZVAD in IMDM-complete). The cultures were returned to the incubator, after which the media was removed and the cultures were rinsed with cold PBS and placed on ice. PBS was removed and 100 μL of lysis buffer [1% TritonX-100, 10% Glycerol, 2mM EDTA, 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM PMSF, 10% Phosphatase Inhibitor Cocktail (Sigma), 10% Protease Inhibitor Cocktail (Sigma)] was added to each well. Cells were scraped and lysates from 3 individual wells were pooled into one tube. Lysates were incubated on ice for 15 minutes, after which they were frozen at -20°C until use. Protein concentration of total cell lysates was determined by the BCA method. 40 μg of total protein was mixed with SDS-PAGE loading buffer to a final concentration of 10% β-mercaptoethanol, 10% glycerol, 2% SDS, 0.1% Bromophenol blue, 50 mM Tris-HCl, pH 6.8 and boiled for 7 minutes. Insoluble matter was pelleted briefly by centrifugation.

Electrophoresis and Transfer

Denatured lysates were loaded onto a discontinuous SDS-PAGE gel with 10% resolving and 4% stacking gels and electrophoresed at 80V through the stacking gel and 120 V through the resolving gel using a Mini-Protean II system and Tris-glycine electrophoresis buffer [25 mM Tris, 250 mM glycine, 0.1% SDS]. After electrophoresis was complete, the resolved proteins were transferred using a Mini-Protean II system onto PVDF membranes using either 26 mAmps of current for 17 hours or 4 hours at 200 mAmps of current in Towbin's Buffer (39 mM glycine, 48 mM Tris, 20% methanol).

Blocking and Antibody Incubation

Membranes were rinsed for 5 minutes in TBS buffer [100 mM Tris (pH 7.5) with 140 mM NaCl] containing 0.1% Tween-20 (TBST) and subsequently blocked in 5% skim milk powder dissolved in TBST for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibody dilution (**Table 3.2**) in either 5% skim milk powder dissolved in TBST or 5% BSA dissolved in TBST. The next morning, membranes were then washed 3 times 15 minutes each in TBST on a rocking horizontal shaker and then probed with an appropriate secondary antibody conjugated to HRP (diluted in 5% milk/TBST) for 1.5 hours at room temperature. Following this, membranes were washed for 3 times 10 minutes each. Membranes were developed using enhanced chemiluminescence reagent [Amersham Pharmacia Biotech, Buckinghanshire, UK] on X-ray films. Membranes were subsequently stripped in stripping buffer (100 mM β-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris-HCl, pH 6.6) for 30 minutes at 50°C with gentle agitation and rinsed in large volumes of TBST 2 times for 10 minutes each. Individual membranes were re-blocked and probed up to 3 times with antibodies of different molecular weight. All membranes were probed with α-actin to control for total protein loading.

3.5 Densitometry of Western Blots

Following X-ray development with ECL reagent, films were scanned using an Epson scanner at 3200 dpi resolution. Densitometry (density per square millimeter) was analyzed using Quantity One imaging software (Bio-Rad). The density of the bands was subtracted from the background density. Each band was normalized to the density of α-actin staining for comparison between protein expression in each lane.

Table 3.2. Antibody suppliers and dilutions.

Antibody	Dilution	Company	Species
Phospho-p38 MAPK (Thr180/Tyr182)	1:1000	Cell Signaling	Mouse (monoclonal)
p-38 MAPK (total)	1:1000	Cell Signaling	Rabbit (polyclonal)
Phospho-p44/42 (ERK1/2) Thr202/Tyr204	1:1000	Cell Signaling	Rabbit (polyclonal)
p44/42 (ERK1/2) total	1:1000	Cell Signaling	Rabbit (polyclonal)
α-Actin	1:10,000	Chemicon	Mouse
Phospho-SAPK/JNK(Thr183/Tyr185)	1:1000	Cell Signaling	Rabbit
Syncytin (HERV-W)	1:1000	Gift	Mouse
Glial Cell Missing-1	1:1000	Aviva	Rabbit
phospho-MEK1/2 (Ser217/221)	1:1000	Cell Signaling	Rabbit
MEK1/2 (47E6)	1:1000	Cell Signaling	Rabbit (Monoclonal)
Anti-caspase 8	1:1000	Medical and Biological Laboratories	Mouse
Anti-caspase 3	1:2000	Stressgen	Rabbit

3.6 Immunofluorescent Staining-Desmoplakin

After 5 days in culture, media was removed from the culture and the cells were rinsed with PBS. Cells were fixed with acetone:methanol (1:1) for 10 minutes at -20°C and rehydrated with PBS. Non-specific staining was blocked with 10% normal goat serum in PBS for 1 hour at room temperature. Anti-desmoplakin antibody [IgG_1 monoclonal antibody, 10μg/ml; ICN ImmunoBiologicals, Costa Mesa, CA] was diluted 1:100 (10 μg/ml) in antibody diluent (Dakocytomation) and incubated overnight at 4°C. Wells were washed 3 times 2 minutes each with PBS and then secondary antibody [Alexa Fluor goat anti-mouse IgG (H+L) 488 conjugate (Molecular Probes)] was added at a dilution of 1:500 in antibody diluent for 1 hour at room temperature. Nuclei were stained with 4′,6-diamino-phenylindole (DAPI; 100 μl of 1.4 μg/ml; Molecular Probes) for 10 minutes at room temperature. Wells were then washed 3 times for 2 minutes each with PBS.

3.7 Immunofluorescent Staining- Placental Alkaline Phosphotase

After 5 days in culture, media was removed from the culture and the cells were rinsed with PBS. Cells were fixed with 4% Paraformaldehyde for 10 minutes at room temperature and rehydrated with PBS. Non-specific staining was blocked with 10% normal goat serum in PBS for 1 hour at room temperature. Anti-Placental Alkaline phosphatase antibody [IgG2a monoclonal antibody, clone 8B6, 10 μg/ml; Sigma, St. Louis, MO] was used at 2 ug/ml, diluted in antibody diluent, and incubated overnight at 4°C. Wells were washed 3 times 2 minutes each with PBS and then incubated with 1μg/ml Alexa Fluor goat anti mouse (H+L) 546 conjugate [Molecular Probes] antibody in the dark at room temperature for 1.5 hours. Wells were washed with PBS 3 times for 2 minutes each.

3.8 Immunofluorescent Staining-TUNEL

After 5 days in culture, media was removed from the culture and the cells were rinsed with PBS. Cells were fixed with acetone:methanol (1:1) for 10 minutes at -20°C and rehydrated with PBS. Fixed cultures were preincubated with TdT buffer [30 mM Tris-base, pH 7.2; 140 mM sodium cacodylate; 1mM cobalt chloride] for 10 minutes at room temperature while TUNEL reaction mixture was prepared. TUNEL reaction (5/μl TdT, 16.5 μM dATP, 16.5 μM bio-16-dUTP in TdT buffer) was carried out at 37°C for 1 hour. The reaction was stopped by adding 2X SSC [300 mM NaCl; 30mM sodium citrate] for 10 minutes. The fixed cells were washed once with PBS to neutralize the pH. Non-specific staining was blocked with 10% normal goat serum in PBS for 1 hour at room temperature. Biotinylated d-UTP was detected by incubation with 1:500 dilution of Alexafluor 546 Strepavidin conjugate. Nuclei were stained for 10 minutes with DAPI (100 μl of 1.4 μg/ml; Molecular Probes) and unbound antibody and dye were removed by washing 3 times 2 minutes each in PBS.

3.9 Immunofluorescent Staining-GCM-1

After 2, 3, 4 and 5 days in culture, media was removed from the culture and the cells were rinsed with PBS. Cells were fixed with acetone:methanol (1:1) for 10 minutes at -20°C and rehydrated with PBS. Non-specific staining was blocked with 10% normal

goat serum in PBS for 1 hour at room temperature. After blocking solution was removed, a 1:500 dilution of GCM-1 antibody (Aviva) in antibody diluent was added to the wells and incubated overnight at 4°C. Unbound antibody was removed by washing 3 times 2 minutes each in PBS. GCM-1 expression was detected by incubation with 1:500 dilution of Alexafluor 546 goat anti rabbit conjugate in antibody diluent for 1 hour at room temperature. Nuclei were stained for 10 minutes with DAPI and unbound antibody and dye were removed by washing 3 times for 2 minutes each in PBS.

3.10 Visualization of Immunofluorescent Staining, Digital Photography and Analysis

Images were obtained using an Olympus 1X2-UCB motorized inverted research microscope equipped with a Lambda DG-4 high-speed filter changer and a Roper Scientific Cascade 16 bit digital monochrome camera (Olympus, Melville, NY) using a 10 power long range objective. Digital images of each well (four fields per well) were obtained with DAPI- 350 nm wavelength (blue), Cy3-546 nm wavelength (red), or FITC-488 nm wavelength (green) filters. Slidebook 3.0 software [Carsen, Markham, Ontario, Canada] was used to capture and export the images. Multinucleation was determined by manual counting of digital images of nuclei/desmoplakin-stained cells using Adobe Photoshop software. Placental alkaline phosphatase staining intensity was analyzed using Image Pro-Plus (Media Cybernetics, Del Mar, CA) setting isotype control (IgG_{2a}) treated wells as zero and the relative intensity of the fluorescent areas was calculated.

3.11 Quantitation of Syncytialization

After dual exposure of desmoplakin/DAPI stained cells to FITC (488 nm) and DAPI (350 nm) wavelengths, pictures were exported and manually assessed. Total nuclei per field at 10 x magnification were assessed. In addition, the total number of nuclei present in syncytia (defined as ≥2 DAPI-stained nuclei per desmoplakin-delineated area) was recorded. The syncytialization rate was determined by the calculation of total syncytialized nuclei/total nuclei present to give % syncytializated nuclei. Conversion from nuclei present in 10 power field to nuclei present in well was done using the average number of nuclei per field and multiplying by 297.56 (area of 96 well plate surface=0.33

cm^2 or 33 mm^2 and area of field under microscope at 10 X= calculated as follows: 1 nuclei=10 uM=17 pixels, area of field= 652 by 492 pixels or 383.53 μm by 289.4 μm which gives an area of 0.1109 mm^2 conversion factor to go from 0.1109 mm^2 to 33 mm^2 is 33mm^2 /0.1109 mm^2 or 297.56).

3.12 Quantitation of Apoptosis

After dual exposure of TUNEL positive/DAPI stained cells to Cy3 (546 nm) and DAPI (350 nm) wavelengths, pictures were exported and manually assessed. Total nuclei per field at 10 x magnification were assessed. In addition, the total number of nuclei fluorescing red (TUNEL positive) were recorded. The apoptosis rate was determined by the calculation of total TUNEL positive nuclei/total nuclei present to give % apoptotic nuclei. Conversion from nuclei present in 10 power field to nuclei present in well was done using the average number of nuclei per field and multiplying by 297.56 (area of 96 well plate surface=0.33 cm^2 and area of field under microscope at 10 X= 0.1109 mm^2).

CHAPTER 4: RESULTS

4.1 Patient Population Comprising Study Groups.

Placentae were collected after delivery from three study groups, normal (n=14), IUGR (n=14) and IUGR-PE (n=3) with patient characteristics found in **Table 4.1**. Statistical significance was determined using the non-parametric Kruskal-Wallis test comparing medians due to the small numbers of IUGR-PE placentae. We separated IUGR and IUGR-PE initially because we wanted to explore the differences between IUGR and normal and felt that the preeclamptic condition may further confound our results. Among the three study groups, maternal age was significantly different with mothers of normal infants being significantly older (30.5 ± 4.9 years) than those mothers bearing IUGR (25 ± 5.2 years) or IUGR-PE (19 ± 4.9 years) babies. Maternal smoking was more prevalent in mothers bearing IUGR babies (50% smokers) compared to one of three smoking mothers in the IUGR-PE group and one of fourteen in the normal group. As smoking is a risk factor for IUGR, exclusion of placentae from smoking mothers would have limited our sample size. Amongst the three groups, blood pressure was only significantly higher in the IUGR-PE group. Only women with preeclampsia experienced proteinuria. All of the babies born in the normal and IUGR-PE groups were delivered via caesarian section as were 9 of 14 babies in the IUGR group. The gestational age in the normal group was significantly later than IUGR or IUGR-PE groups with a median gestational age of 38.8 ± 0.6 weeks compared to 36.6 ± 2.8 weeks (IUGR) and 34 ± 2.64 weeks (IUGR-PE). The gestational age was significantly lower in IUGR; however the median age of IUGR infants was still near to term. The birthweight was significantly greater in the normal group (3215 ± 623 grams) compared to the IUGR (2182 ± 637 grams) and IUGR-PE (1340 ± 650 grams). All of the babies born in the normal group were between the 10th and 90th centiles for their gestational age, all of the IUGR and IUGR-PE babies being born below the 10th percentile. The gender distribution amongst the normal group was 50% male/50% female, but higher numbers of male babies were born in the IUGR (9 of 14) and IUGR-PE (2 of 3) groups. All of the babies in the normal group had normal Doppler flow as well as normal amniotic fluid levels. In contrast, all of

the babies born in the IUGR or IUGR-PE study groups had either abnormal umbilical Doppler flow, low amniotic fluid levels or asymmetric growth. Of the placental weights recorded, there was a significant difference between the three groups with normal babies having heavier placentae (598 ± 49 grams; n=5) than the IUGR (353.5 ± 157 grams; n=6) and IUGR-PE (245.9 grams; n=1) groups.

4.2 Trophoblast apoptosis is increased in IUGR after 5 days under standard culture conditions.

Previous studies in our lab have shown that at 20% oxygen (standard culture conditions), normal and IUGR trophoblasts exhibit the same amount of apoptosis within the first 24 hours of culture, indicating CT apoptosis is unchanged between normal and IUGR. Apoptosis of trophoblasts remaining in culture after 5 days was measured as the fraction of nuclei having double-stranded DNA nicks as visualized by TUNEL analysis. However, after 5 days of culture at 20% oxygen, I found that apoptosis was significantly greater when compared by an unpaired t test ($p<0.05$) in IUGR trophoblast cultures (values expressed as mean ± standard deviation: 15.3 ± 4.8% TUNEL positive nuclei) than in normal trophoblast cultures (5.8 ± 1.7% TUNEL positive nuclei) (**Figure 4.1**).

Table 4.1. Patient characteristics of normal, IUGR and IUGR-PE study groups. Values presented are means ± standard deviation and statistieal significance was determined by the Kruskal Wallis test (which compared the medians) for non-parametric data with $p<0.05$ versus normal (*) and $p<0.05$ IUGR versus IUGR-PE (#) considered significant.

	Normal (n=14)	IUGR (n=14)	IUGR-PE (n=3)
Maternal Age	30.7 ± 4.9	25.1 ± 5.2*	21.3 ± 4.9*#
Maternal Smoking	1/14	7/14*	1/3
Maternal Blood Pressure(mmHg):			
Systolic	119 ± 6.7	123 ± 9.1	160 ± 21*#
Diastolic	78 ± 6.7	75± 8.5	98 ± 10 *#
Proteinuria	negative	negative	All >2+*#
Mode of Delivery:			
Vaginal	0	5	0
Ceasarian	14	9	3
Primiparous	2/14	4/14	3/3*#
Gestational Age (weeks)	38.6 ± 0.62	36.1 ± 2.8*	34 ± 2.64*#
Birth weight (g)	3443 ± 623	2034 ± 637*	1592± 650*#
Birth weight Centile:			
10th to 90th %	14	0*	0*
5th to 10th %	0	7	0
<5th %	0	7	3
Sex:			
Male	7/14	9/14	2/3
Female	7/14	5/14	1/3
Abnormal Doppler Flow (ADF)	0/14	6/14*	1/3*
Oligohydramnios (OH) (Fluid <7 cm)	0/14	6/14*	3/3*
Asymmetric Growth (AG) (Head/abdominal circumference <1.2)	0/14	11/14*	3/3*
Placental weight (g)	603 ± 49 (n=5)	361 ± 157* (n=6)	245.9 (n=1)

4.3 Trophoblast differentiation as assessed by syncytialization, hCG and hPL production is increased in IUGR but not in IUGR-PE.

Trophoblast differentiation in culture has been extensively studied in our lab. Our cultures of >99.9% pure CT can spontaneously differentiation over a period of time, with maximal differentiation (as assessed by peak hCG production) seen at day 5 of culture under standard culture conditions (100). Trophoblast differentiation was assessed using three markers: percentage of syncytialized nuclei, production of human chorionic gonadotropin, and production of human placental lactogen. Supernatants from normal, IUGR and IUGR-PE trophoblast cultures were collected after Day 1, Day 3 and Day 5, after which cultures were fixed and stained for desmoplakin to delineate cellular boundaries and DAPI to visualize the nuclei. Percent syncytialization of trophoblast cultures was determined by taking the average number of nuclei contained within a syncytium (>2 nuclei/desmoplakin-delineated area) and dividing by the average total number of nuclei per field followed by multiplying by 100. Production of hCG and hPL was assessed in Day 1, Day 3 and Day 5 supernatants by high sensitivity ELISA (detection limit for β-hCG: <1mU/ml; for hPL: 0.3μg/ml), the sum of the three values being the total hCG or hPL produced over the 5 day period. Hormone production was normalized to the number of nuclei remaining in the well (10^4 nuclei/well).

IUGR trophoblast cultures exhibit significantly higher ($p<0.05$) amounts of syncytialization (mean ± standard deviation: 32.4 ± 7.6%) than either normal (15.7 ± 6.8%) or IUGR-PE (13.1 ± 0.6%) trophoblast cultures after 5 days (**Figure 4.2**-upper panel [summary and representative picture of desmoplakin staining from each group]). Similarly, IUGR trophoblasts produce significantly higher ($p<0.05$) levels of hCG (30.6 ± 18.8 mU/10^4 nuclei) and hPL (0.51 ± 0.28 μg/10^4 nuclei) than either normal (hCG: 11.0 ± 5.9 mU/10^4 nuclei; hPL: 0.24 ± 0.18 μg/10^4 nuclei) or IUGR-PE (hCG: 5.5 ± 4.2 mU/10^4 nuclei; hPL: 0.08 ± 0.11 μg/10^4 nuclei) trophoblast cultures (**Figure 4.2**-center and bottom panels).

Taken together, these observations suggest that IUGR trophoblast cultures undergo greater rates of apoptosis and differentiate more than normal trophoblast cultures and that IUGR trophoblast cultures and IUGR-PE trophoblast cultures are fundamentally different with IUGR-PE cultures behaving as normal in culture. As I wanted to explore

the difference between IUGR and normal, I excluded IUGR-PE placentae from the rest of this study.

4.4 Elevated trophoblast apoptosis and differentiation in IUGR is blocked by neutralizing antibody to TNFα.

Previously, our lab has shown that elevated apoptosis after 24 hours in IUGR trophoblast cultures can be blocked by addition of neutralizing antibody to TNFα (115). Upon addition of excess neutralizing antibody to TNFα for the 5 day culture period, IUGR trophoblast cultures exhibited a significant ($p<0.05$) decrease in the percentage of TUNEL positive nuclei (from 14.0 ± 5.0% TUNEL positive nuclei versus 8.0 ± 4.1% in IgG_1 isotype control) while normal trophoblast cultures remained unchanged (5.8 ± 1.7% versus 5.6 ± 0.9% in isotype control) (**Figure 4.3**). This indicates that either IUGR trophoblast cultures are more sensitive to TNFα levels than normal trophoblast cultures or that IUGR trophoblasts are producing more TNFα than normal cultures. Indeed, after addition of neutralizing antibody to TNFα, IUGR trophoblast cultures had more nuclei remaining after 5 days in culture than isotype treated controls although this failed to reach significance (**Figure 4.4**-bottom panel).

When trophoblast differentiation markers were assessed after addition of neutralizing antibody to TNFα, I found that elevated levels of syncytialization in IUGR were blocked (mean ± standard deviation: from 30.0 ± 5.6% [isotype control] to 18.4 ± 3.7% [neutralizing antibody to TNFα]) while syncytialization was slightly stimulated in normal trophoblast cultures (from 17.5 ± 5.9% [isotype control] to 23.1 ± 8.4% [neutralizing antibody to TNFα]) (**Figure 4.4**-top panel). Interestingly, inhibition of excess syncytialization was only seen when antibody to TNFα was added at plating (data not shown), indicating that the environment in which the CT attach to the plate influences differentiation markers later on in culture. Similarly, antibody to TNFα also decreased the amount of hCG produced in IUGR trophoblast cultures (mean ± standard deviation: 45.8 ± 29.6 $mU/10^4$ nuclei [isotype control] versus 28.5 ± 11.6 $mU/10^4$ nuclei [neutralizing antibody to TNFα]) to levels near normal (19.6 ± 3.3 $mU/10^4$ nuclei [isotype control] versus 18.0 ± 3.0 $mU/10^4$ nuclei [neutralizing antibody to TNFα]) (**Figure 4.4**-centre panel). Contrary to previous findings using the BeWo cell line,

neutralizing antibody to IL-1β in a dose calculated to neutralize 50 ng/ml of IL-1β did not have a significant effect on either normal (17.5 ± 5.9% [isotype control] versus 15.0 ± 0.1% [neutralizing antibody to IL-1β]) or IUGR (30.0 ± 5.6% [isotype control] versus 27.6 ± 7.9% [neutralizing antibody to IL-1β]) trophoblast syncytialization. Similarly, neutralizing antibody to IL-1β did not significantly impact the production of hCG by either normal (19.6 ± 3.3 $mU/10^4$ nuclei [isotype control] versus 12.4 ± 3.0 $mU/10^4$ nuclei [neutralizing antibody to IL-1β]) or IUGR (45.8 ± 29.6 $mU/10^4$ nuclei [isotype control] versus 51.7 ± 22.4 $mU/10^4$ nuclei [neutralizing antibody to IL-1β]) trophoblast cultures (**Figure 4.4**-centre panel). In BeWo cells, inhibition of IL-1β signaling through neutralizing antibody decreased hCG production, however, IL-1β signaling inhibition had no effect on either normal or IUGR primary trophoblast cultures.

4.5 TNFα is able to activate the phosphorylation of p38 MAPK, ERK1/2, JNK and caspase 3 cleavage.

TNFα has been shown in other epithelial cells to activate the phosphorylation of the MAPKS (p38, ERK1/2 and JNK) as well as the activation of the caspase cascade including caspase 8 and caspase 3 cleavage. Addition of 10 and 80 ng/ml of TNFα for 10 minutes to 3 different normal trophoblast cultures (24 hour old cultures-majority of cells as CT) resulted in the phosphorylation of p38 MAPK (**Figure 4.5-a,b**). Stimulation of CT cultures with 10 ng/ml of EGF for 10 minutes also stimulated the phosphorylation of p38 MAPK which served as a positive control as EGF has previously been shown to activate p38 MAPK phosphorylation (100).

Addition of TNFα to CT cultures (which are 24 hours old) for 10 minutes also stimulates the phosphorylation of ERK1/2 (**Figure 4.6**). Again, EGF was used as a positive control as our lab has previously demonstrated EGF activation of ERK1/2 phosphorylation (100). In agreement with data previously reported, I found that addition of 10 ng/ml of EGF stimulated ERK1/2 phosphorylation versus untreated controls (**Figure 4.6-a,b**). Similarly addition of 10 and 80 ng/ml of TNFα to CT cultures for 10 minutes resulted in an increase in ERK1/2 phosphorylation versus untreated controls. ERK1/2 phosphorylation by TNFα was inhibited by two hour pretreatment with the

specific inhibitor PD098059 which prevents the upstream activity of MEK1/2 thus preventing phosphorylation of downstream ERK1/2 (**Figure 4.6-b,d**).

TNFα was also able to phosphorylate JNK (**Figure 4.7**). Treatment of 3 different normal CT cultures with 10 ng/ml of TNFα resulted in strong phosphorylation of JNK, much higher than the weak signal stimulated by addition of 10 ng/ml of EGF.

TNFα binding to TNFR1 on the surface of epithelial cells triggers caspase 8 and caspase 3 cleavage (49). As such, I hypothesized TNFα would stimulate the cleavage of both caspase 8 and 3 in trophoblast cultures. Treatment of two different normal CT cultures with 10 ng/ml of TNFα for 6, 12 and 18 hours did not result in significant upregulation of caspase 8 cleavage (**Figure 4.8-a,b,c**). In contrast, when CT lysates were assessed by caspase 3 cleavage after treatment with TNFα for 18 hours, significant cleavage was seen (**Figure 4.8-d**). As caspase 8 cleavage remained undetectable by western blotting, I hypothesized that CT cultures were responding to the TNFα challenge by increasing the expression of an anti-apoptotic protein (such as Bcl-2 or Mcl-1). To counteract this anti-apoptotic protein expression, I used the protein synthesis inhibitor cyclohemixide to prevent any protein expression. When CT cultures were treated with 10 μg/ml of cycloheximide for 18 hours, caspase 3 cleavage was stimulated (**Figure 4.8-d**) but caspase 8 cleavage remained undetectable (**Figure 4.8-c**). Similarly, when CT cultures were treated with 10 ng/ml of TNFα for 18 hours, caspase 3 cleavage was stimulated (**Figure 4.8-d**) but caspase 8 cleavage remained undetectable (**Figure 4.8-c**). When protein synthesis was inhibited at the same time cells were exposed to 10 ng/ml of TNFα, there were increased amounts of caspase-3 cleavage (**Figure 4.8-d**). TNFα induced cleavage of caspase 3 was inhibited by 20 μM of ZVAD even in the presence of protein synthesis inhibition (**Figure 4.8-d**). There appears to be small amounts of caspase 8 cleavage at 6, 12, or 18 hours with or without TNFα treatment, but ZVAD did not reduce this (**Figure 4.8-a, b, c**).

4.6 Addition of exogenous TNFα stimulates apoptosis and cell loss in normal and IUGR trophoblasts.

Our lab has previously shown that TNFα is able to stimulate trophoblast apoptosis in normal cultures (35, 70). I also have found that TNFα is able to stimulate apoptosis as

assessed by % TUNEL positive nuclei on day 5 in normal trophoblasts (n=2) in a dose dependent manner (**Figure 4.9**) but does not have a significant effect on IUGR trophoblasts (n=4). In normal trophoblast cultures, the lowest dose of TNFα (0.01 ng/ml) did not have any significant effect on trophoblast apoptosis (mean ± standard deviation: 7.2 ± 0.7% TUNEL positive nuclei [control] versus 6.7 ± 0.1% TUNEL positive nuclei [0.01 ng/ml TNFα]). However, with increasing doses of TNFα, there are increasing numbers of TUNEL positive nuclei (10.3 ± 0.2% TUNEL positive nuclei [0.1 ng/ml TNFα] to 13.4 ± 0.6% TUNEL positive nuclei [1 ng/ml TNFα] to 11.4 ± 0.2% TUNEL positive nuclei [10 ng/ml TNFα]). Statistical significance as determined by 2-way ANOVA followed by Bonferroni post-tests showed that only the 1 ng/ml dose of TNFα had significantly higher amounts of apoptosis on Day 5 than untreated normal trophoblast control cultures. In contrast, IUGR trophoblast cultures (n=4) did not exhibit increased apoptosis on Day 5 of culture upon exposure to increasing doses of TNFα compared to untreated control cultures (mean ± standard deviation of % TUNEL positive nuclei: 15.2 ± 4.8% [control]; 13.1 ± 2.9% [0.01 ng/ml TNFα]; 12.7 ± 3.5% [0.1 ng/ml TNFα]; 16.5 ± 1.7% [1 ng/ml TNFα]; and 14.2 ± 2.0% [10 ng/ml TNFα]).

As apoptosis on Day 5 was a 'snapshot' of the effects of TNFα on cell survival, I also assessed the number of nuclei remaining in each well after treatment with TNFα. Increasing doses of TNFα caused increasing cell loss over the 5 days in culture in both normal and IUGR trophoblasts (**Figure 4.10**). In normal trophoblast cultures (n=9), control ($43.9 \times 10^3 \pm 9.6 \times 10^3$ nuclei/well), 0.01 ng/ml TNFα ($46.4 \times 10^3 \pm 11.6 \times 10^3$ nuclei/well) and 0.1 ng/ml TNFα ($40.4 \times 10^3 \pm 7.2 \times 10^3$ nuclei/well) treatments did not significantly affect the number of cells remaining; however, 1 ng/ml TNFα ($29.8 \times 10^3 \pm 5.0 \times 10^3$ nuclei/well) and 10 ng/ml TNFα ($31.5 \times 10^3 \pm 1.1 \times 10^3$ nuclei/well) caused a significant ($p<0.05$) decline in the number of nuclei remaining after 5 days. In IUGR trophoblast cultures (n=10), only the highest dose of TNFα caused a significant ($p<0.05$) decrease in the number of nuclei remaining after 5 days ($27.0 \times 10^3 \pm 7.6 \times 10^3$ nuclei/well) compared to control ($41.4 \times 10^3 \pm 1.5 \times 10^3$ nuclei/well), 0.01ng/ml TNFα ($43.2 \times 10^3 \pm 15.6 \times 10^3$ nuclei/well), 0.1 ng/ml TNFα ($34.9 \times 10^3 \pm 11.2 \times 10^3$ nuclei/well) or 1 ng/ml TNFα ($31.5 \times 10^3 \pm 4.3 \times 10^3$ nuclei/well) treatments (**Figure**

4.10). Thus, although TNFα did not significantly increase apoptosis in normal trophoblast cultures at a concentration of 10 ng/ml, it did induce nuclei loss.

4.7 Addition of exogenous TNFα stimulates syncytialization in normal trophoblasts in a dose dependent manner.

Previously I showed that neutralizing antibody to TNFα prevented both increased apoptosis and increased differentiation in IUGR trophoblasts (**Figure 4.3 and 4.4**). Addition of exogenous TNFα was shown to stimulate trophoblast syncytialization in normal trophoblast cultures (n=5) but did not affect IUGR trophoblast cultures (n=8) (**Figure 4.11-a**). In untreated controls, normal trophoblast cultures had significantly ($p<0.05$) less syncytialization (17.5 ± 6.0%) than IUGR trophoblast cultures (29.8 ± 5.8%). The significant difference between the groups was maintained with the 0.01 ng/ml TNFα treatment (normal: 25.0 ± 9.5%; IUGR: 34.3 ± 8.5%). However, with 1 ng/ml (25.6 ± 7.2%) and 10 ng/ml (27.8 ± 5.7%) TNFα treatments trophoblast syncytialization became significantly different from the control treatment in normal cultures (**Figure 4.11-a**). At the higher doses of TNFα, the significance between the groups was lost (normal: 27.0 ± 10.9%; IUGR: 35.1 ± 8.0% [0.1ng/ml TNFα], normal: 25.9 ±7.2%; IUGR: 35.3 ± 11.3% [1 ng/ml TNFα], and normal: 27.8 ± 5.7%; IUGR: 31.7 ± 4.5% [10 ng/ml TNFα]). When I expressed total nuclei number present as ST and CT (in contrast to percentage of syncytialized nuclei), I found that increasing dose of TNFα significantly decreased the number of ST nuclei in IUGR trophoblast cultures alone at 10 ng/ml and decreased the number of CT nuclei in both normal at 1 ng/ml and 10 ng/ml and IUGR at 10 ng/ml (**Figure 4.11-b**).

4.8 Exogenous TNFα suppresses hCG production in both normal and IUGR trophoblasts.

Neutralization of endogenous TNFα action by addition of neutralizing antibody suppressed the elevated levels of hCG seen in IUGR trophoblast cultures. This suggested that endogenous TNFα was able to stimulate hCG production because when endogenous TNFα was neutralized, hCG production was inhibitied in IUGR trophoblast cultures. As such, I wanted to determine if exogenous TNFα could stimulate trophoblast hCG

production. Contrary to what I expected, exogenous TNFα suppressed hCG production in both normal and IUGR trophoblast cultures in a dose dependent manner (**Figure 4.12**). In normal trophoblast cultures (n=5), hCG production was decreased slightly by addition of 0.01 ng/ml TNFα (mean ± standard deviation: 8.8 ± 4.7 mU/10^4 nuclei) compared to the control (12.6 ± 5.8 mU/10^4 nuclei). However, with addition of 0.1 ng/ml of TNFα, hCG production significantly decreased to 4.0 ± 3.8 mU/10^4 nuclei TNFα and was even further decreased with addition of 1 ng/ml TNFα (1.0 ± 0.9 mU/10^4 nuclei)and 10 ng/ml of TNFα (0.3 ± 0.3 mU/10^4 nuclei). In IUGR trophoblast control cultures (n=8), there was significantly more hCG produced (20.4 ± 10.2 mU/10^4 nuclei) compared to normal control cultures (12.6 ± 5.8 mU/10^4 nuclei) and this was unaffected by addition of 0.01 ng/ml TNFα (21.7 ± 10.7 mU/10^4 nuclei). Similarly to normal trophoblast cultures, addition of 0.1 ng/ml of TNFα significantly decreased hCG production by IUGR trophoblast cultures (12.4 ± 8.8 mU/10^4 nuclei) and this was further decreased by addition of 1 ng/ml (3.3 ± 3.2 mU/10^4 nuclei) and 10 ng/ml (3.1 ± 2.9 mU/10^4 nuclei) of TNFα.

4.9 Inhibition of p38α and ERK1/2 MAPKs has differential effects on normal and IUGR trophoblasts.

Previously, our lab has shown that EGF signaling through p38 MAPK stimulates both hCG production and syncytialization in normal trophoblast cultures (101). As I previously showed TNFα to stimulate p38 MAPK phosphorylation, I used an inhibitor of p38 MAPK to determine if endogenously produced TNFα was mediating the elevated differentiation in IUGR trophoblast cultures. SB203580 is a specific inhibitor of the α and β isoforms of p38 MAPK. SB202474 is a negative control compound to be used with SB203580. When normal trophoblast cultures (n=7) are exposed to the vehicle (0.1% DMSO), there is significantly less ($p<0.05$) syncytialization (16.0 ± 6.2%) than in IUGR trophoblast cultures (n=8) (29.2 ± 7.8%) exposed to the vehicle control (**Figure 4.13**). The negative control compound SB202474 (10μM) did not significantly alter syncytialization in normal (15.3 ± 4.4%) or IUGR (24.7 ± 4.5%) trophoblast cultures. In contrast, the specific p38 MAPKα inhibitor, SB203580 (10μM), significantly ($p<0.05$) decreased both normal (7.3 ± 2.6%) and IUGR (15.5 ± 2.6%) trophoblast

syncytialization. In fact, syncytialization in IUGR with p38 MAPK inhibition was the same as that of normal trophoblast control cultures.

Other research has shown both p38 MAPK and ERK1/2 to be involved in trophoblast differentiation (51). As I previously demonstrated that TNFα was able to activate ERK1/2 phosphorylation, I used an inhibitor of ERK1/2 phosphorylation to determine if endogenous TNFα was involved in the elevated differentiation seen in IUGR trophoblast cultures. PD098059 is a specific inhibitor of the activity of MEK1/2, the upstream enzyme which phosphorylates ERK1/2. Inhibition of ERK1/2 with PD098059 (10μM) did not affect normal trophoblast (n=7) syncytialization (16.0 ± 6.2% [control] versus 16.9 ± 4.7% [PD098059]) but significantly decreased syncytialization in IUGR trophoblast cultures (n=8) (29.2 ± 7.8% [control] versus 20.1 ± 8.1% [PD098059]). When both ERK1/2 and p38 MAPKα are inhibited with 10μM each of PD098059 and SB203580, syncytialization is slightly more reduced in normal (5.7 ± 2.3%) compared to SB203580 alone (7.3 ± 2.6%) but is significantly lower (9.1 ± 6.0%) than inhibition with either PD098059 (20.1 ± 8.1%) or SB203580 (15.5 ± 2.6%) alone in IUGR trophoblast cultures (**Figure 4.13-upper panel**).

The production of hCG appears to be differentially regulated by ERK1/2 and p38 MAPK in normal and IUGR trophoblasts. As seen before (**Figure 4.12**) only this time with vehicle (0.1% DMSO), normal trophoblast cultures (n=7) produced significantly less ($p<0.05$) hCG (mean ± standard error of the mean (SEM): 12.5 ± 1.4 mU/10^4 nuclei) than IUGR trophoblast cultures (n=8) (45.5 ± 10.9 mU/10^4 nuclei) exposed to the vehicle (**Figure 4.13-lower panel**). When the negative control compound SB202474 (10 μM) was added to normal trophoblast cultures, there was a slight (11.4 ± 1.3 mU/10^4 nuclei) but not significant decrease in the amount of hCG produced compared to the control (12.5 ± 1.4 mU/10^4 nuclei). However, in IUGR trophoblast cultures, addition of the negative control compound SB202474 significantly decreased the amount of hCG produced over the five day period (16.5 ± 6.7 mU/10^4 nuclei) compared to control (45.5 ± 10.9 mU/10^4 nuclei) indicating that the analogue compound to the p38 MAPKα inhibitor has some non-specific inhibitory effect in IUGR trophoblast cultures which may be more sensitive to the presence of a foreign compound than normal trophoblast cultures. Addition of the p38 MAPK specific inhibitor SB203580 resulted in complete suppression of hCG

production in both normal (0.13 ± 0.06 mU/10^4 nuclei) and IUGR (0.63 ± 0.45 mU/10^4 nuclei) trophoblast cultures. In contrast, addition of ERK1/2 inhibitor PD098059 resulted in a significant decrease in hCG production in IUGR trophoblast cultures (24.8 ± 7.0 mU/10^4 nuclei) alone but did not have an effect on normal trophoblast cultures (13.2 ± 1.9 mU/10^4 nuclei). Inhibition of both ERK1/2 and p38 MAPK phosphorylation resulted in complete suppression of hCG production in both normal (0.17 ± 0.06 mU/10^4 nuclei) and IUGR (0.16 ± 0.07 mU/10^4 nuclei) trophoblast cultures.

Cellular survival over the 5 day period was assessed by quantification of the total number of nuclei remaining in each well. I found that there were significantly fewer nuclei remaining in the IUGR trophoblast cultures (mean ± SEM: 32.7 ± 6.1 x 10^3 nuclei/well) than in the normal cultures (44.6 ± 6.0 x 10^3 nuclei/well) exposed to 0.1% DMSO (vehicle) (**Figure 4.14**). The number of nuclei remaining was not significantly affected by any of the treatments, but there was a trend towards increased nuclei loss in the presence of ERK1/2 inhibition with PD098059.

4.10 The pan-caspase inhibitor ZVAD does not inhibit elevated differentiation seen in IUGR.

Previous work using placental explants has shown caspase-8 to be involved and required for CT differentiation into ST as well as hCG production (16). I previously found that TNFα was able to stimulate caspase-3 cleavage (a caspase which is downstream of caspase-8) and that caspase-3 was not cleaved in the presence of 20 μM of the pan-caspase inhibitor ZVAD (**Figure 4.8**). As TNFα is known to activate the caspase cascade and increased TNFα signaling appears to mediate elevated trophoblast differentiation in IUGR trophoblasts (both increased syncytialization [**Figure 4.4-a**] and hCG production [**Figure 4.4-b**]), I hypothesized that inhibition of caspase activity with the pan-caspase inhibitor ZVAD would decrease elevated differentiation seen in IUGR trophoblast cultures. Contrary to my hypothesis and the placental explant work, inhibition of caspases with ZVAD had no effect on either trophoblast differentiation marker (syncytialization- **Figure 4.15-upper panel** or hCG production-**Figure 4.15-centre panel**) in both normal and IUGR trophoblast cultures. As found previously, normal trophoblast cultures (n=8) had significantly less syncytialization (mean ± SEM:

20.3 ± 2.9%) than IUGR trophoblast cultures (n=7) (31.2 ± 2.4%) exposed to 0.1% DMSO (vehicle) in addition to significantly less hCG production (normal (n=10): 14.1 ± 2.3 mU/10^4 nuclei; IUGR (n=9): 29.5 ± 8.5 mU/10^4 nuclei) over the 5 day period. These two markers of trophoblast differentiation were unaffected by inhibition of caspase cleavage as both syncytialization in normal (27.2 ± 1.6%) and IUGR (34.2 ± 8.1%) trophoblast cultures as well as hCG production in normal (9.0 ± 2.3 mU/10^4 nuclei) and IUGR (20.2 ± 9.7 mU/10^4 nuclei) cultures were not significantly altered by the presence of ZVAD.

Surprisingly, inhibition of caspase activity also failed to impact trophoblast survival (**Figure 4.15-bottom panel**). Treatment with ZVAD did not significantly increase the number of nuclei remaining over the 5 day period in either normal ([mean ± SEM] control: 48.5 ± 3.6 x 10^3 nuclei/well; ZVAD: 51.0 ± 16.6 x 10^3 nuclei/well) or IUGR trophoblast (control: 44.9 ± 4.6 x 10^3 nuclei/well; ZVAD: 48.3 ± 7.2 x 10^3 nuclei/well) cultures although there was a slight trend towards increased survival with slightly higher numbers of nuclei present in the ZVAD treatment group. However, addition of ZVAD to cultures treated with TNFα suppressed the TNFα induced cell loss (**Figure 4.17**) indicating that the ZVAD was still functional.

4.11 JNK inhibition has no significant effect on syncytialization or hCG in normal trophoblasts.

Our lab has previously shown that the JNK MAPKs are important in trophoblast survival signaling (141). As TNFα is able to activate the phosphorylation of JNK and appears to be increased in IUGR as neutralizing antibody to TNFα blocks elevated differentiation in IUGR, I wanted to determine if JNK 1 and 2 were involved in trophoblast differentiation. Initial experiments were done using concentrations of 10 μM JNK II inhibitor, but massive cell death (~95% death by Day 4 in culture) resulted in necessitating a decreased concentration of inhibitor. Previously, our lab used a concentration of 90 nM and found it to significantly decrease cell survival (141). Compared to vehicle (0.1% DMSO) control [mean ± SEM: 21.5 ± 2.2%], syncytialization was unchanged in normal trophoblast cultures (n=4) when JNK II negative control compound (90 nM) [23.1 ± 3.7%] or JNK II inhibitor (SP600125- 90 nM) [23.7 ± 2.2%]

was added (**Figure 4.16-top panel**). JNK II inhibitor in combination with SB203580 (10 μM) significantly reduced (8.6 ± 1.0%) syncytialization as did JNK II inhibitor in combination with PD098059 (10 μM) (11.8 ± 2.2%), which was in contrast to the lack of inhibition seen with PD098059 alone in normal trophoblast cultures (**Figure 4.13-top panel**). The presence of all three MAPK inhibitors (SB203580, PD098059 and JNK II inhibitor) had no further effect on syncytialization (9.5 ± 0.8%).

The effect of JNK II inhibition on hCG production was largely non-existent. Compared to 0.1% DMSO control (vehicle) (20.3 ± 11.4 mU/10^4 nuclei), the addition of JNK II negative control (20.0 ± 9.4 mU/10^4 nuclei) and JNK II inhibitor (20.9 ± 8.4 mU/10^4 nuclei) itself did not have a significant impact on hCG production (**Figure 4.16-centre panel**). However, addition of the p38 inhibitor SB203580 prevented all hCG production (0.05 ± 0.05 mU/10^4 nuclei), similar to its effects alone (**Figure 4.13-centre panel**). Dual inhibition of JNK II (with SP600125) and ERK1/2 (with PD098059), in contrast to their effect on syncytialization (**Figure 4.16-top panel**), did not significantly decrease hCG production (19.8 ± 6.5 mU/10^4 nuclei). Addition of all three MAPK inhibitors abolished hCG production (0.07 ± 0.04 mU/10^4 nuclei), probably attributable to p38 MAPKα inhibition. Trophoblast survival was unaffected by inhibition of JNK II at 90 nM concentration with and without the presence of p38 MAPK or ERK1/2 inhibition (**Figure 4.16-bottom panel**).

4.12 Exogenous TNFα has differential effects on trophoblast differentiation markers which are mediated by p38 MAPK and ERK1/2.

As exogenous TNFα was able to stimulate syncytialization in normal trophoblast cultures (**Figure 4.11**), but suppressed hCG production (**Figure 4.12**). I wanted to determine which pathway TNFα was acting through to stimulate syncytialization. Since neutralizing antibody to TNFα only decreased syncytialization and hCG production in IUGR when it was present during plating (the first four hours of culture), I also wanted to determine if adding TNFα during the first four hours of culture would impact trophoblast differentiation markers. My hypothesis was that TNFα addition at plating would stimulate syncytialization and hCG production and TNFα present during the entire culture period would stimulate syncytialization but decrease hCG production. I found

that when compared to control cultures (n=5) (mean ± SEM: 18.5 ± 2.0%), normal trophoblast syncytialization was increased (although not significantly) by a dose of 1 ng/ml TNFα added for the first four hours of culture (24.6 ± 1.7%) (**Figure 4.17**). When 1 ng/ml of TNFα was present for the duration of culture (at washing to Day 5), syncytialization was significantly greater than the control (27.4 ± 3.0%). Addition of ERK1/2 inhibitor (PD098059) in addition to TNFα decreased TNFα stimulated syncytialization back to control levels (17.5 ± 2.1%). Addition of p38 MAPKα inhibitor (SB203580) suppressed TNFα induced syncytialization (6.3 ± 0.5%) and brought syncytialization down to levels seen with SB203580 inhibition alone (7.3 ± 2.6% - **Figure 4.13**). To confirm that TNFα was not mediating increased syncytialization through the activation of caspases, I used the inhibitor ZVAD (20 μM) and found that it did not inhibit the elevated syncytialization seen with TNFα treatment (27.2 ± 0.7%).

The production of hCG over the 5 day culture period was assessed and I found those trophoblast cultures exposed to 1 ng/ml TNFα for the first four hours of plating were able to produce the same amount of hCG (23.3 ± 5.0 mU/10^4 nuclei) compared to control cultures (22.4 ± 6.0 mU/10^4 nuclei) (**Figure 4.17**). In contrast, the presence of 1 ng/ml TNFα for the entire culture period (from washing to Day 5) resulted in suppression of hCG production (2.4 ± 1.2 mU/10^4 nuclei). TNFα induced suppression of hCG production was slightly less when ERK1/2 was inhibited (6.8 ± 2.3 mU/10^4 nuclei). However, addition of p38 MAPK inhibitor (SB203580) resulted in complete suppression of hCG production (0.0 ± 0.0 mU/10^4 nuclei). ZVAD was unable to prevent the TNFα induced inhibition of hCG production (3.4 ± 1.2 mU/10^4 nuclei).

Cellular survival was assessed after 5 days in culture in the presence or absence of TNFα with and without inhibitors PD098059, ZVAD and SB203580 by determining the average total number of nuclei remaining in each well. I found that addition of 1 ng/ml of TNFα to normal trophoblast cultures for 4 hours at plating slightly decreased (49.1 ± 6.9 x10^3 nuclei/well) the number of nuclei remaining compared to the untreated control (44.6 ± 5.3 x10^3 nuclei/well). However, the presence of 1 ng/ml of TNFα for 5 days significantly decreased the number of nuclei remaining on day 5 (30.5 ± 3.0 x10^3 nuclei/well). Addition of the ERK1/2 inhibitor (PD098059) did not prevent TNFα induced nuclei loss (29.7 ± 5.6 x10^3 nuclei/well). The p38 MAPK inhibitor (SB203580)

slightly increased the number of nuclei remaining (37.4 ± 2.0 x10^3 nuclei/well) but ZVAD completely prevented the TNFα induced nuclei loss (48.0 ± 6.2 x10^3 nuclei/well).

4.13 Trophoblast differentiation markers (syncytialization and hCG production) are differentially regulated by changes in oxygen tension.

Previous trophoblast differentiation work in our lab has been done under standard culture conditions (oxygen tension ~138 mmHg). We have previously shown that hCG production is initially absent and reaches a peak at day 5 or 6 of culture, corresponding with maximal syncytialization induced by EGF (101) under standard conditions. As trophoblast differentiation *in vivo* does not occur at 138 mmHg of oxygen and is more likely occurring at oxygen tensions of between 38 mmHg (5%) and mmHg (8%) (115), I wanted to confirm that elevated differentiation seen in IUGR was also reflected in conditions normoxic (5%) for the placenta. I chose three oxygen tensions: 1) standard culture conditions or hyperoxia (~138 mmHg or 18% oxygen); 2) low-normoxia (~38 mmHg or 5% oxygen); and 3) hypoxia (~15 mmHg or 2% oxygen). Also, as IUGR placentae appear to have a wider range of oxygen tensions within the intervillous space (167), I felt it was important to verify the role oxygen may play in trophoblast differentiation. **Figure 4.18** shows representative images of desmoplakin staining of one normal and one IUGR trophoblast culture on day 5 under three different oxygen tensions. I found that syncytialization was not changed by oxygen tension and that IUGR trophoblast cultures (n=6) (mean ± SEM: 31.1 ± 2.3%) had significantly more syncytialization compared to normal trophoblast cultures (n= 6) (23.7 ± 2.9%) on Day 5 (**Figure 4.19-upper panel**) under standard conditions. This held true at the lower two oxygen tensions: IUGR trophoblast cultures have significantly more syncytialization at 5% (33.1 ± 2.2%) and 2% oxygen (29.0 ± 1.9%) than normal trophoblast cultures at 5% (24.4 ± 2.7%) and 2% (25.6 ± 2.7%).

In contrast to syncytialization, oxygen tension dramatically altered hCG production in both normal and IUGR trophoblast cultures by day 5 of culture (**Figure 4.19-centre panel**). At standard culture conditions (hyperoxia), IUGR trophoblast cultures produced significantly more hCG (60.8 ± 11.0 mU/10^4 nuclei) than normal trophoblast cultures (17.0 ± 2.1 mU/10^4 nuclei). In contrast, IUGR trophoblast cultures

under 5% oxygen had significantly less hCG production (6.6 ± 1.9 mU/10^4 nuclei), which was even lower than normal trophoblast cultures (12.1 ± 2.4 mU/10^4 nuclei). This was further suppressed by conditions of hypoxia (2% oxygen) in which hCG production was abolished in both IUGR (0.9 ± 0.5 mU/10^4 nuclei) and normal (0.5 ± 0.3 mU/10^4 nuclei) trophoblast cultures.

Previous work done in primary trophoblast cultures suggests that low oxygen is able to stimulate trophoblast proliferation. I found that the number of nuclei remaining under the three oxygen tensions were dependent on the placenta type and not on the oxygen tension (**Figure 4.19-bottom panel**). Under standard conditions, there were significantly more nuclei remaining in the normal trophoblast cultures (48.9 ± 2.3 x10^3 nuclei/well) than in the IUGR trophoblast cultures (38.4 ± 2.4 x 10^3 nuclei/well). The number of nuclei remaining in each well was slightly higher when trophoblasts were cultured at 5% oxygen (normal: 61.5 ± 4.3 x 10^3 nuclei/well; IUGR: 42.4 ± 5.5 x 10^3 nuclei/well) but this failed to reach significance. Hypoxia did not change the number of nuclei remaining in either normal (52.6 ± 5.0 x 10^3 nuclei/well) or IUGR (32.6 ± 8.2 x 10^3 nuclei/well) trophoblast cultures over the 5 day period.

4.14 cAMP induces differentiation in both IUGR and normal trophoblasts.

Our lab has previously shown that the cell permeable cAMP analogue 8-bromo-cAMP is able to stimulate hCG production in normal trophoblasts (99). Furthermore, it is well known that cAMP is an inducer of trophoblast syncytialization and hCG production (125, 180). To confirm that IUGR trophoblasts were able to undergo cAMP induced differentiation, I treated IUGR and normal trophoblast cultures with 10^{-5} M of 8-bromo-cAMP at standard culture conditions (or 18% oxygen). I found that in untreated controls, there was significantly less syncytialization in normal trophoblast cultures (n=2) (mean ± SEM: 14.1 ± 0.5%) than in IUGR trophoblast cultures (n=2) (32.9 ± 1.7%) (**Figure 4.20-upper panel**). The difference between the groups was lost upon the addition of 8-bromo-cAMP, resulting in nearly 100% syncytialization in both normal (85.6 ± 4.9%) and IUGR (95.8 ± 0.6%) trophoblast cultures.

When I determined the amount of hCG produced per nuclei in normal and IUGR trophoblast cultures, I found that there was significantly more hCG produced in IUGR

cultures (n=6) (38.0 ± 12.4 mU/10^4 nuclei) than in normal cultures (n=5) (20.0 ± 3.7 mU/10^4 nuclei) (**Figure 4.20-lower panel**). Upon addition of 8-bromo-cAMP, there was a significant increase in the amount of hCG produced in both normal (60.9 ± 9.4 mU/10^4 nuclei) and IUGR cultures (94.9 ± 18.7 mU/10^4 nuclei), although the significant difference between normal and IUGR was maintained. Thus, I was able to confirm that IUGR trophoblast cultures were still capable of increased differentiation despite their lack of response to exogenous TNFα.

4.15 cAMP induces nuclear translocation of transcription factor GCM1 in both normal and IUGR trophoblasts.

Previous research has shown that transcription factor GCM-1 expression is localized to villous CT and that expression does not occur in the extravillous trophoblast (8). Others have shown trophoblast GCM-1 expression to be upregulated by cAMP (37). I wanted to determine the cellular pattern of GCM-1 expression in normal trophoblast cultures under standard culture conditions (18% oxygen) in order to confirm that our cultures consisted mainly of villous CT. I found that GCM-1 was expressed in both the cytoplasmic and nuclear compartments of both syncytialized and non-syncytialized trophoblasts (**Figure 4.21**). Upon addition of 8-bromo-cAMP (10^{-5} M) to normal trophoblast cultures, there is an increase in nuclear GCM-1 expression and an increase in the number of GCM-1 positively stained nuclei (**Figure 4.22**), indicating nuclear translocation of GCM-1.

4.16 cAMP upregulates GCM1 but not syncytin protein expression.

Previous work has shown that GCM-1 expression is upregulated by exposure to cAMP (37). When Day 3 mixed CT/ST cultures from normal and IUGR placentae cultured under standard culture conditions (18% oxygen) are evaluated for GCM-1 expression, I found that there was increased nuclear expression of GCM-1 upon exposure to 8-bromo-cAMP (10^{-5}M) (**Figure 4.23**). In addition, upregulation of GCM-1 expression should lead to increased expression of syncytin, a fusigenic protein involved in trophoblast fusion (211). However, there is decreased syncytin expression upon cAMP

treatment in both normal and IUGR when compared to untreated control lysates from day 3 cultures (**Figure 4.24**).

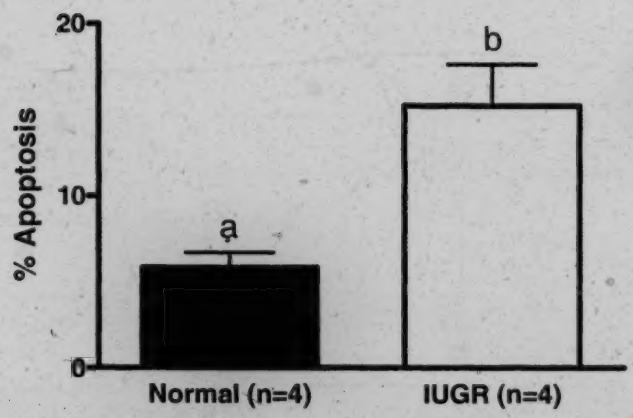


Figure 4.1. The effect of IUGR on trophoblast apoptosis after 5 days under standard culture conditions. IUGR trophoblast cultures have higher numbers of apoptotic nuclei than normal trophoblast cultures after 5 days in 20% oxygen. Values are presented as mean ± standard error of the mean and statistical significance determined by an unpaired t-test with $p<0.05$ being significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

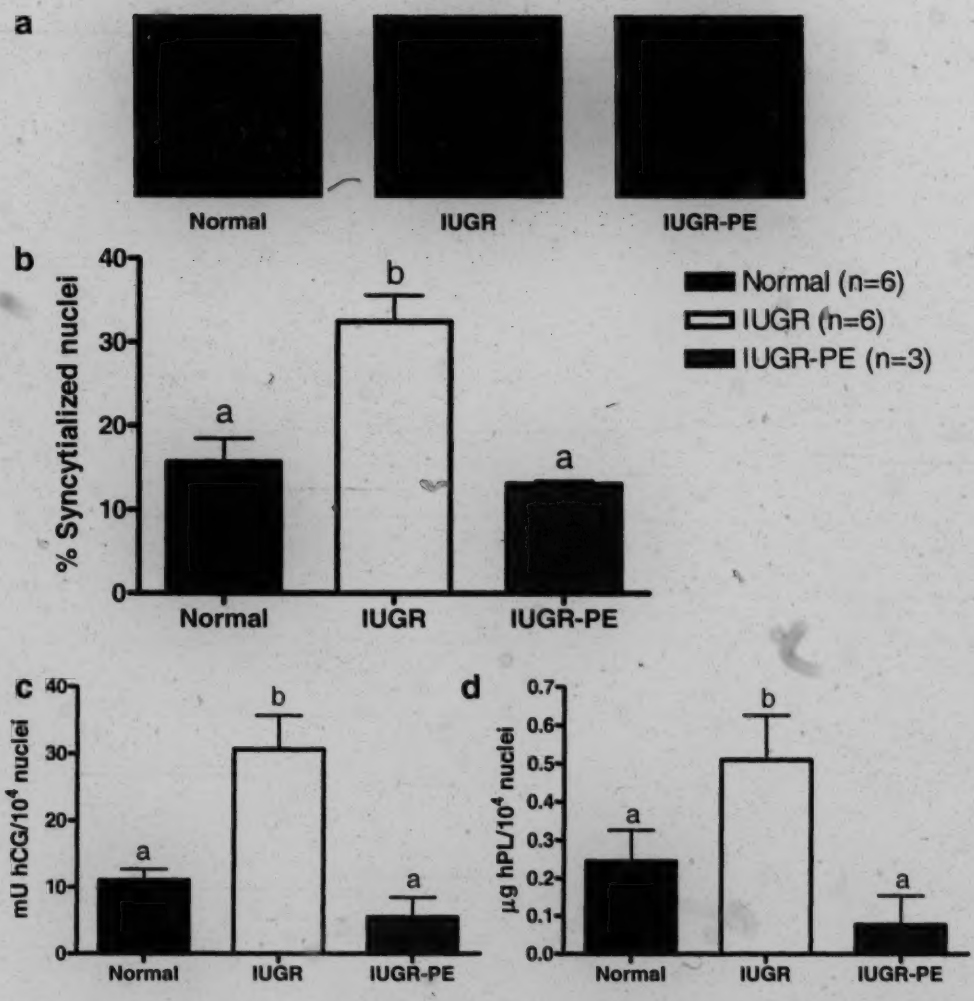


Figure 4.2. The effect of pregnancy complications on three differentiation markers (syncytialization, hCG and hPL) in normal, IUGR and IUGR-PE trophoblasts. Syncytialization (a (representative picture), b), hCG production (c), and hPL production (d) are increased after 5 days in culture in IUGR trophoblasts compared to normal or IUGR-PE trophoblasts. Values are presented as mean ± standard error of the mean and statistical significance determined by 1-way ANOVA with $p<0.05$ being significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

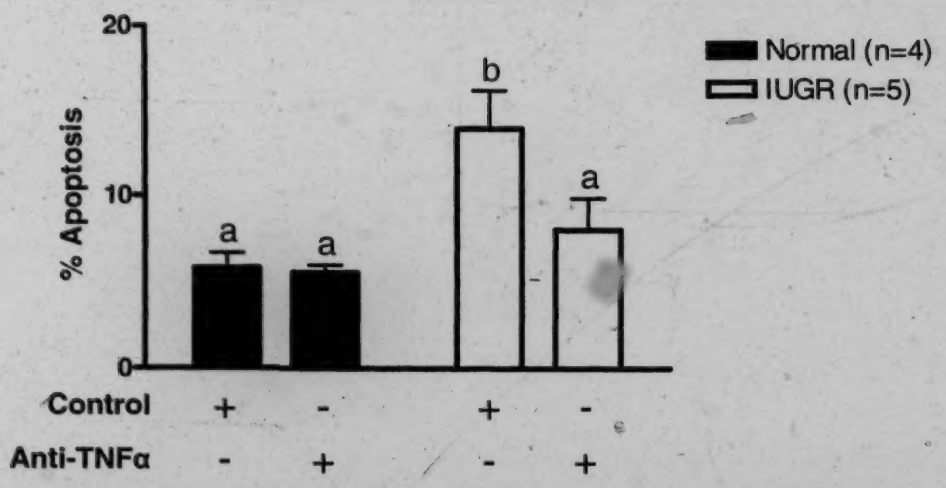


Figure 4.3. The effect of neutralizing antibody to TNFα on trophoblast apoptosis in normal and IUGR cultures. Neutralizing antibody to TNFα suppresses elevated apoptosis in IUGR trophoblast cultures but does not affect normal trophoblast apoptosis. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA followed by Bonferroni post test with $p<0.05$ being significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

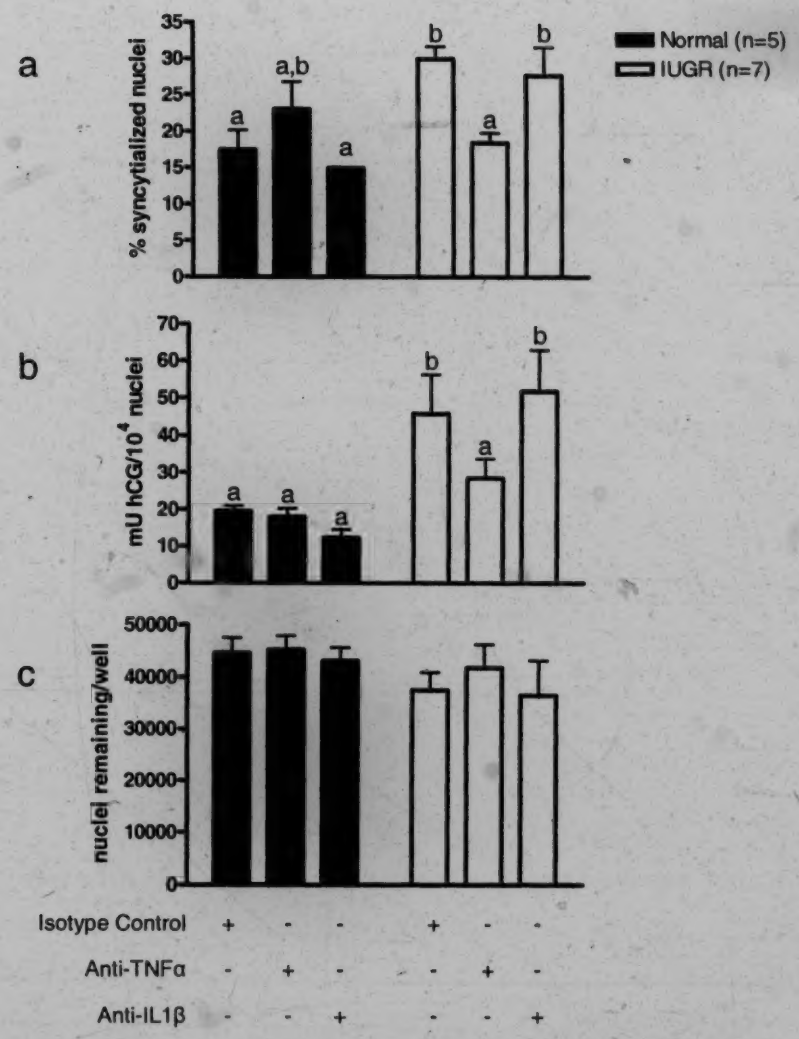


Figure 4.4. The effect of neutralizing antibodies to TNFα and IL1β on syncytialization, hCG production and number of nuclei remaining in normal and IUGR. a) Syncytialization is unaffected by neutralizing antibody to IL1β in both normal and IUGR cultures, but syncytialization significantly decreased in IUGR cultures and slightly increased in normal cultures after addition of neutralizing antibody to TNFα. b) hCG production is unchanged by addition of antibody to IL1β in both normal and IUGR cultures, but neutralizing antibody to TNFα suppresses hCG production in IUGR cultures alone. c) The number of nuclei remaining after 5 days of culture is unchanged by the addition of neutralizing antibodies to TNFα or IL1β. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ being significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

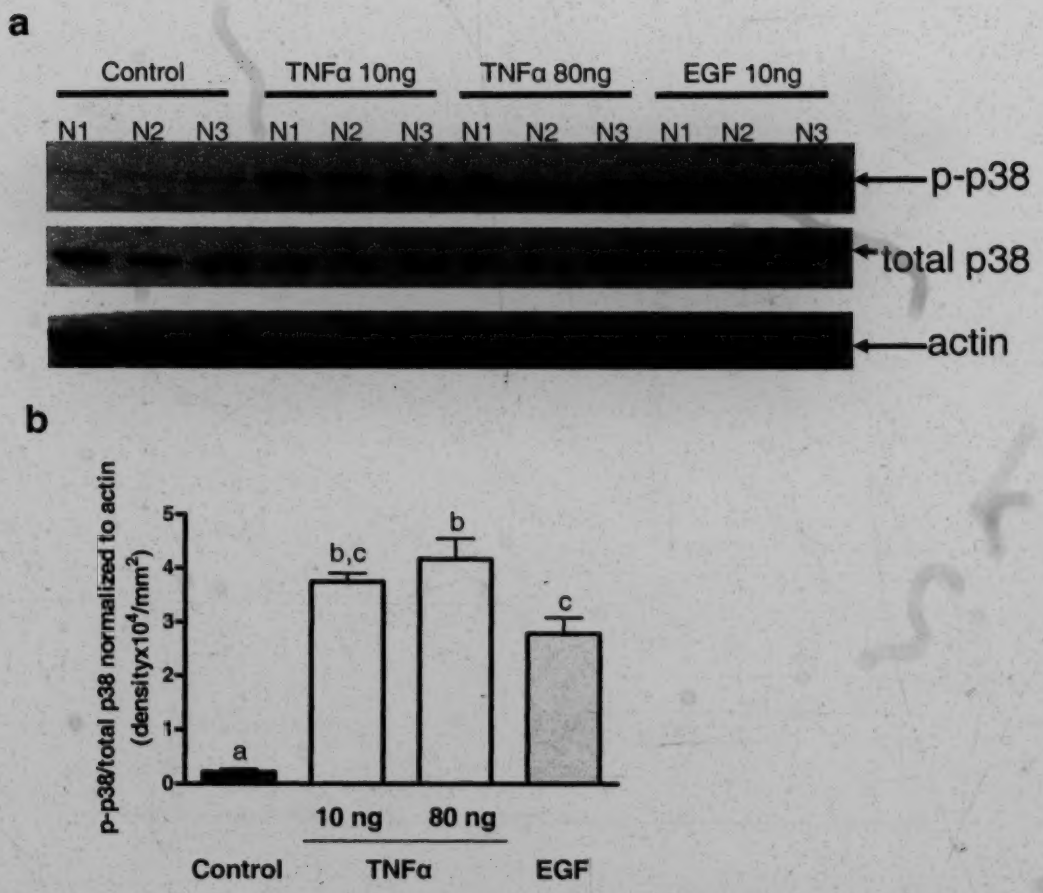


Figure 4.5. The effect of TNFα and EGF on p38 MAPK phosphorylation in normal trophoblast cultures (n=3). a) Expression of phosphorylated p38 MAPK (upper panel) after treatment with control media, two doses (10 ng/ml and 80 ng/ml) of TNFα and EGF (10 ng/ml) for 10 minutes in three different normal trophoblast cultures. TNFα and EGF are both able to stimulate p38 MAPK phosphorylation. The centre panel shows the total amount of p38 MAPK expressed and the bottom panel shows the actin loading control. **b)** Densitometry of the ratio of p-p38 normalized to actin to total p38 normalized to actin with significant differences determined by 1-way ANOVA followed by Tukey's test. EGF and TNFα significantly increased phosphorylation of p38 MAPK.

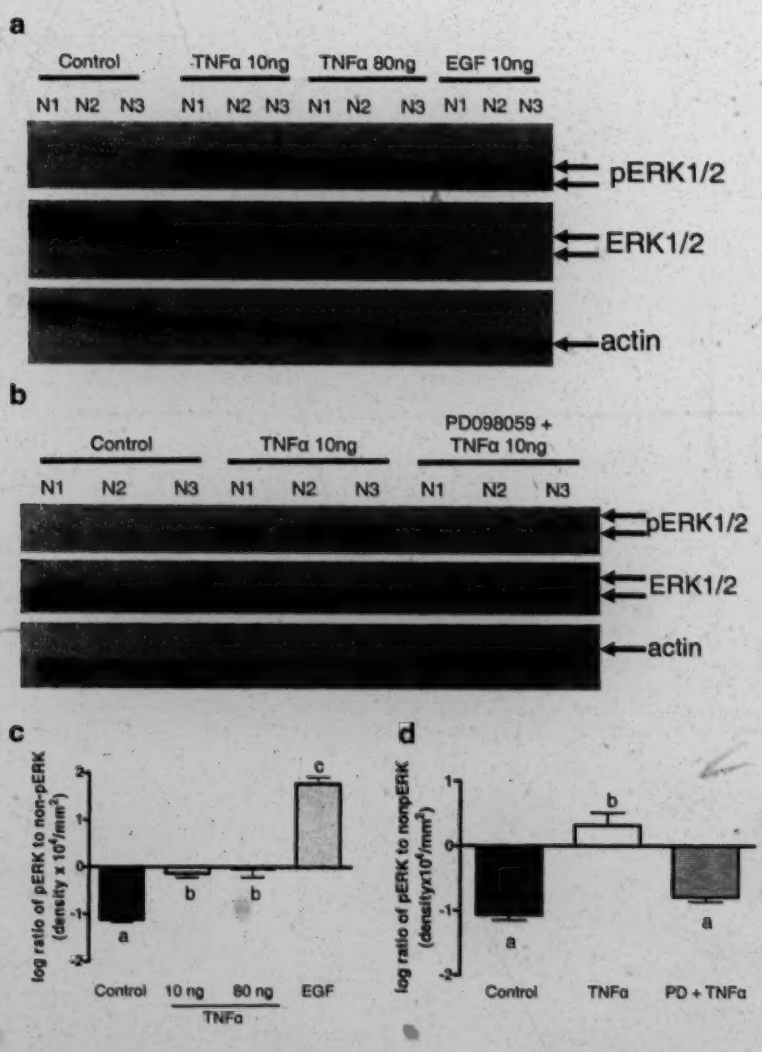


Figure 4.6. The effect of TNFα, EGF and ERK1/2 inhibitor PD098590 on ERK1/2 phosphorylation in normal trophoblast cultures (n=3). a) Expression of phosphorylated ERK1/2 in three different normal trophoblast cultures (upper panel) is stimulated by 10 minute exposure to 10 ng/ml or 80 ng/ml of TNFα as well as 10 ng/ml of EGF. The centre panel shows non-phosphorylated ERK1/2 and the lower panel shows the actin loading control. **b)** Phosphorylation of ERK1/2 is stimulated by 10 ng/ml of TNFα and inhibited by pretreatment with PD098059, a MEK1/2 inhibitor which prevents phosphorylation of ERK1/2. The centre panel shows the non-phosphorylated ERK1/2 pool and the lower panel represents the total cellular protein loaded as actin expression. **c,d)** Densitometry of the log ratio of pERK1/2 normalized to actin to non-phosphorylated ERK1/2 normalized to actin with significant differences determined by 1-way ANOVA followed by Tukey's test. **c)** TNFα significantly increased the ratio of pERK1/2 to ERK1/2, ERK1/2 phosphorylation being even greater with EGF. **d)** TNFα significantly increased ERK1/2 phosphorylation which was inhibited by PD098059.

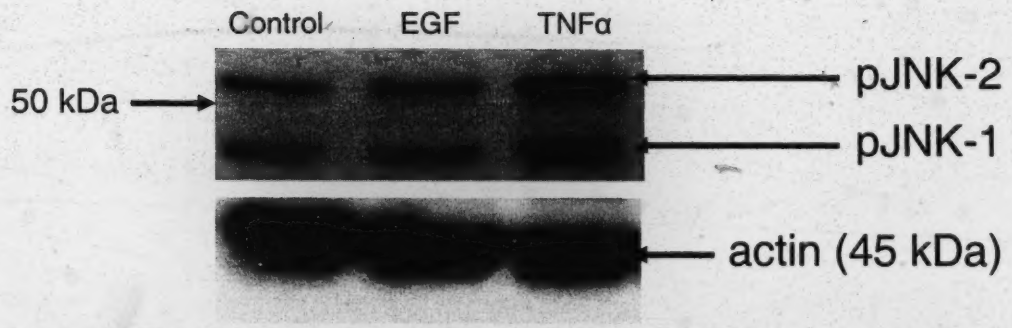


Figure 4.7. The effect of EGF and TNFα on JNK1 and JNK2 phosphorylation. Treatment with 10 ng/ml of EGF did not significantly stimulate JNK1/2 phosphorylation, however, treatment with 10 ng/ml of TNFα increased JNK1/2 phosphorylation in relation to the total amount of protein in normal trophoblast CT culture lysate.

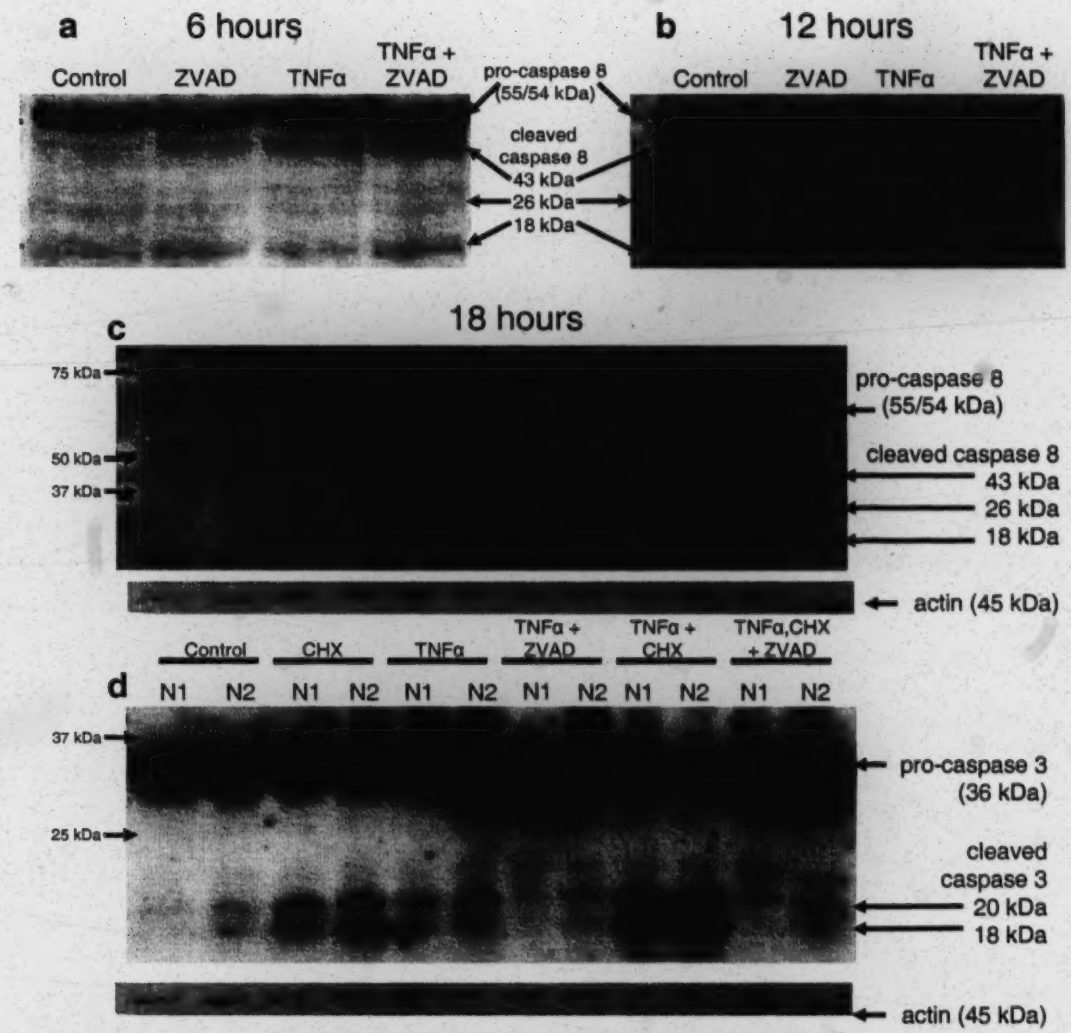


Figure 4.8. The effect of TNFα, ZVAD and cycloheximide on caspase 8 (a, b, and c) and caspase 3 cleavage in normal trophoblast cultures. a) 6 hours after treatment with control media, ZVAD (20 μM), TNFα (10 ng/ml) or TNFα (10 ng/ml) and ZVAD (20 μM) did not alter the amount of caspase 8 cleavage in normal trophoblast CT culture (n=1). **b)** 12 hours after treatment with caspase inhibitor (ZVAD), apoptosis inducer TNFα or the combination of the two treatments did not increase the amount of caspase 8 cleavage in a normal CT culture. **c)** CT cultures (n=2 normals) were exposed to protein synthesis inhibitor cycloheximide (CHX), TNFα (10 ng/ml), TNFα (10 ng/ml) in combination with ZVAD (20 μM) , TNFα with cycloheximide or TNFα, cylcoheximide and ZVAD. Caspase 8 cleavage was largely unchanged following 18 hours of exposure to the treatments. **d)** The same blot from Figure 4.8 c was stripped and probed with antibody to caspase 3. Treatment with cycloheximide and/or TNFα resulted in an increase in caspase 3 cleavage as seen by an increase in the density of the 20 and 18 kDa cleavage products. Treatment with ZVAD (20 μM) prevented both TNFα and cycloheximide induced caspase 3 cleavage.

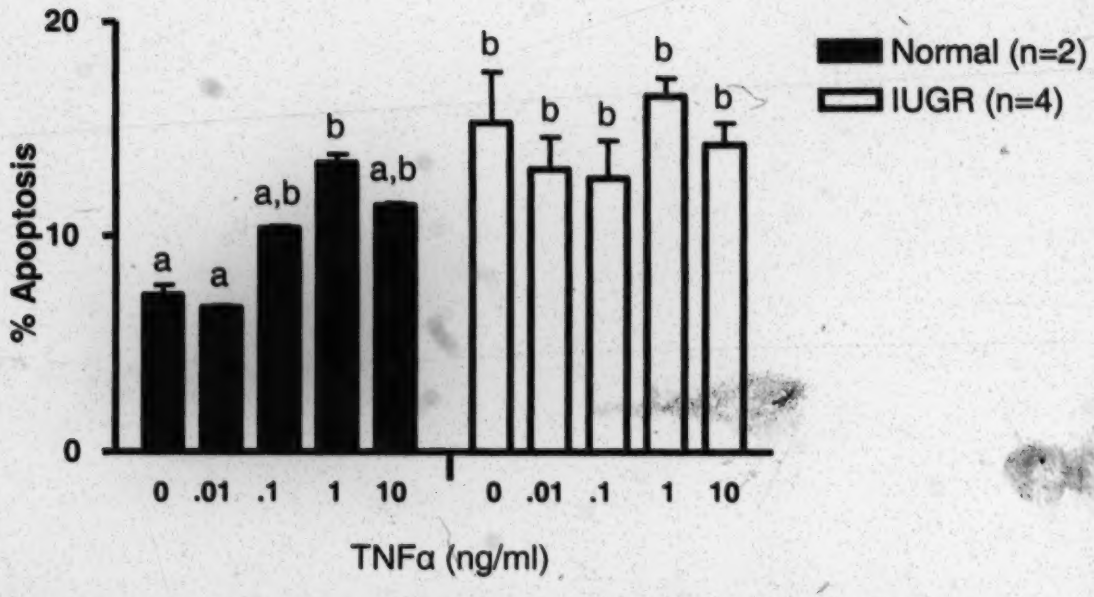


Figure 4.9. The effect of exogenous TNFα dose on trophoblast apoptosis in normal and IUGR cultures after 5 days of exposure. Increasing doses of TNFα significantly increases the percentage of TUNEL positive nuclei in normal trophoblast cultures but has no significant effect on IUGR trophoblasts over a period of 5 days in culture. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ being significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

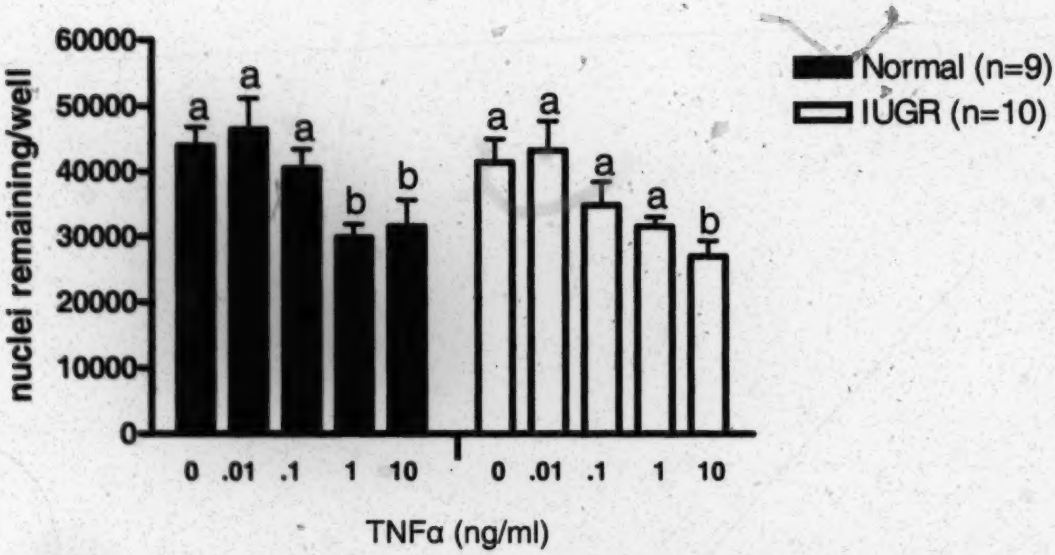


Figure 4.10. The effect of TNFα dose on normal and IUGR trophoblast survival over a 5 day period. Increasing dose of TNFα significantly decreases the number of nuclei remaining after 5 days in culture. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

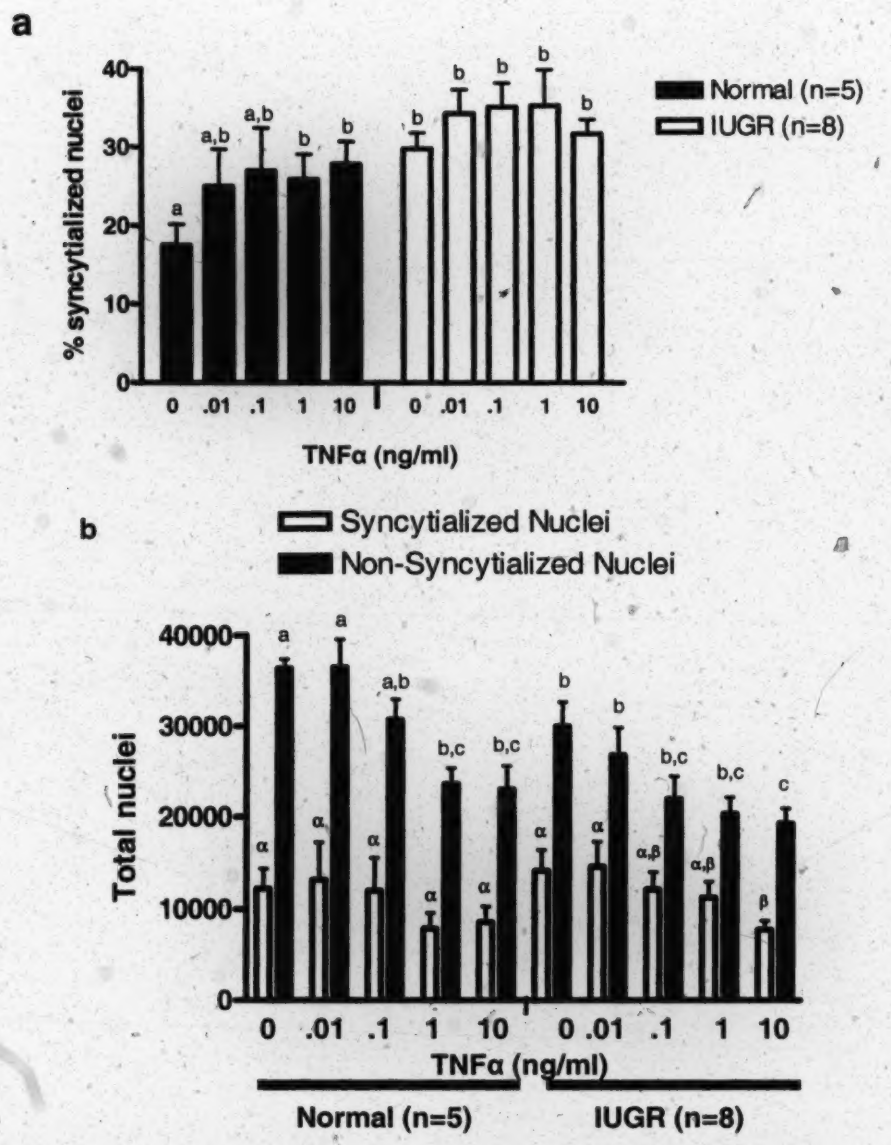


Figure 4.11. The effect of TNFα dose on syncytialization in normal and IUGR trophoblasts. **a)** The effect of TNFα does not react significance in IUGR trophoblasts, but stimulates trophoblast syncytialization in normal trophoblast cultures in a dose dependent manner, attaining significance at 1 and 10 ng/ml. **b)** TNFα dose dependently decreases the number of CT nuclei in both normal (at 1 and 10 ng/ml) and IUGR (at 10 ng/ml) and decreases only IUGR ST nuclei number at 10 ng/ml. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, those letters the same indicate the difference is not significant and different letters indicate significance $p<0.05$.

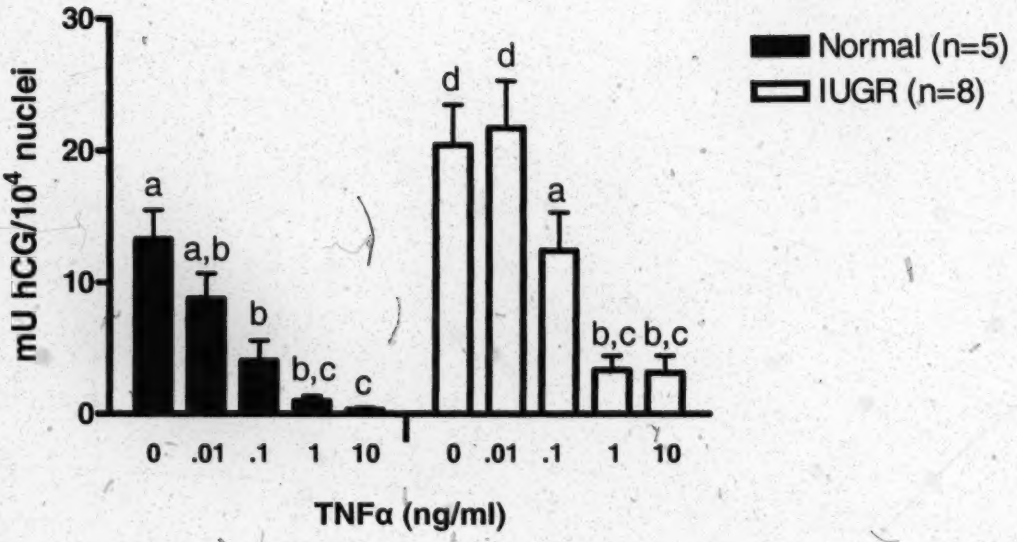


Figure 4.12. The effect of TNFα on hCG production in normal and IUGR trophoblasts. TNFα dose dependently decreases hCG production in both normal and IUGR trophoblasts over a 5 day period in culture. Basal hCG production is higher in IUGR trophoblasts despite the presence of TNFα. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate significance or $p<0.05$.

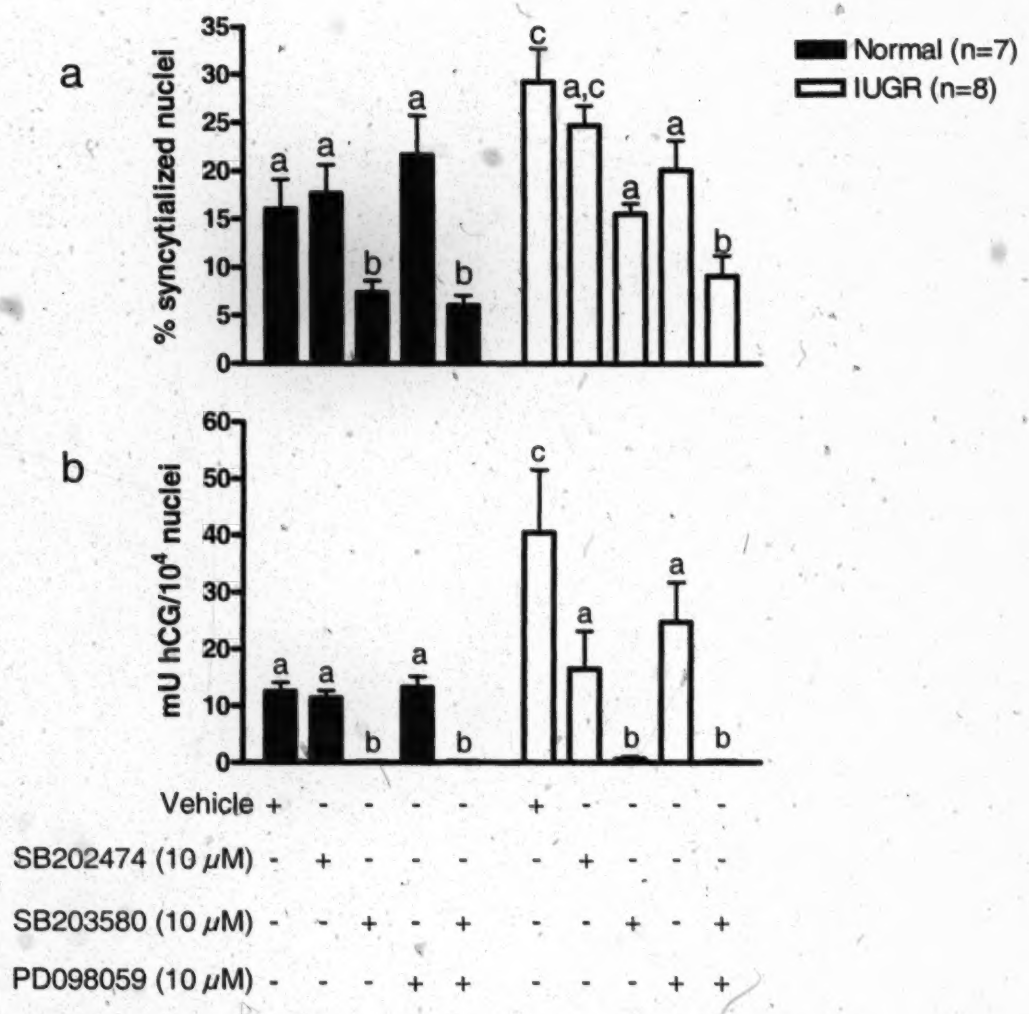


Figure 4.13. The effects of p38 MAPK and ERK1/2 inhibition on normal and IUGR trophoblast syncytialization and hCG production. a) Syncytialization in normal trophoblasts is decreased when p38 MAPK is inhibited with SB203580 but is unaffected by ERK1/2 inhibition with PD098059. In contrast, IUGR trophoblast syncytialization is decreased by both ERK1/2 and p38 MAPK inhibition. b) hCG production in normal and IUGR trophoblasts is dependent on p38 MAPK but in IUGR is also decreased by ERK1/2 inhibition. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

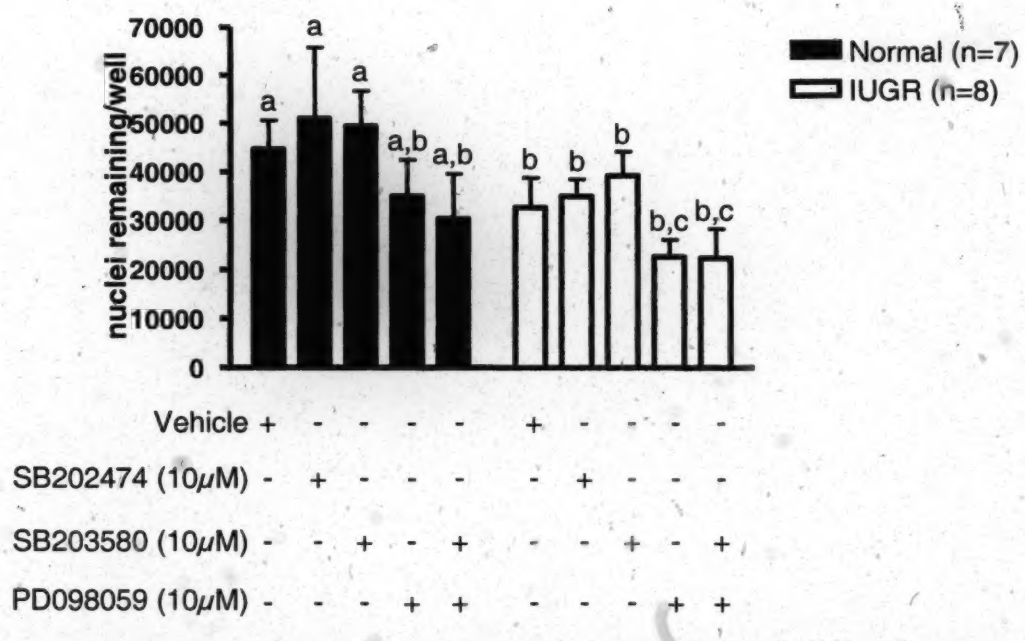


Figure 4.14. The effects of p38 MAPK and ERK1/2 inhibition on normal and IUGR trophoblast survival over 5 days in culture. Fewer nuclei remain after 5 days in culture in IUGR trophoblasts than normal. ERK1/2 inhibition (PD098059) slightly decreases the number of nuclei remaining in both populations of trophoblasts. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

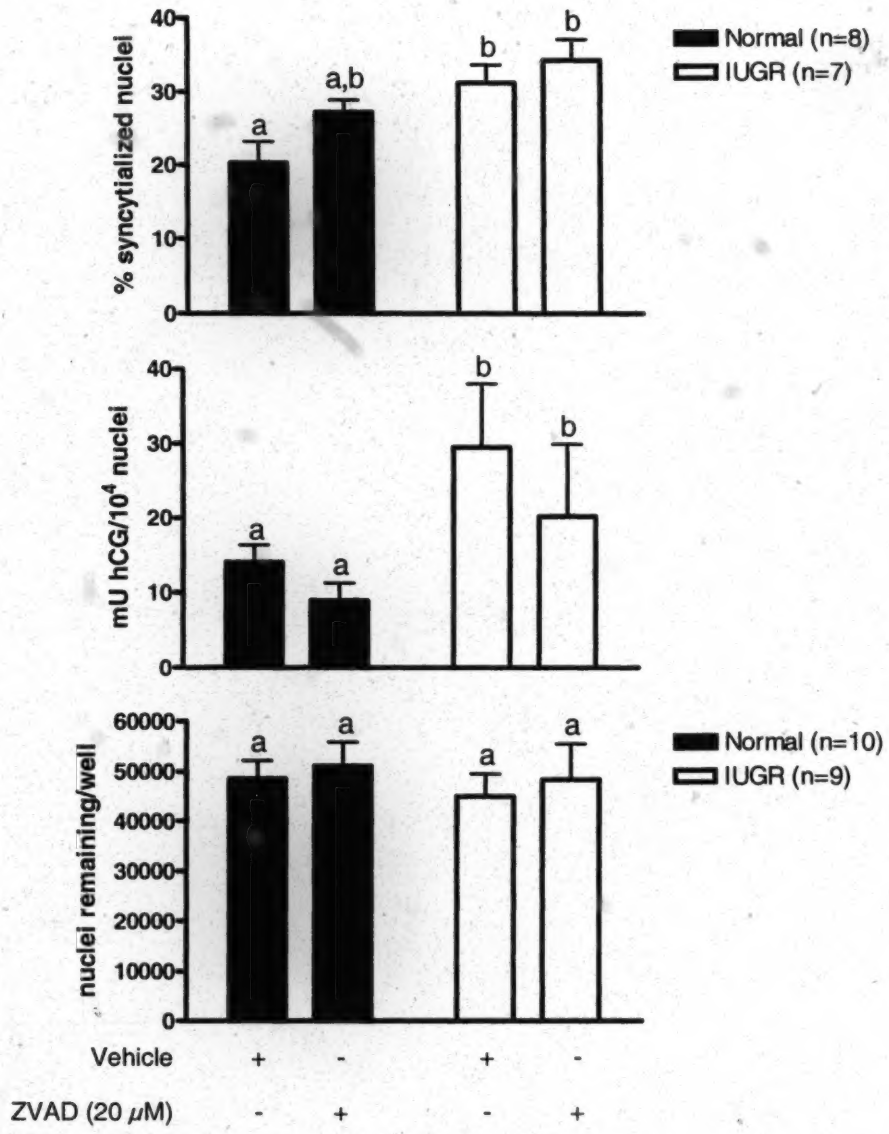


Figure 4.15. The effect of pan-caspase inhibitor ZVAD-fmk on trophoblast differentiation and survival in normal and IUGR. Syncytialization (upper panel), hCG production (centre panel), and cell survival (bottom panel) are not affected by inhibition of caspase activity with ZVAD-fmk in either normal or IUGR trophoblasts. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

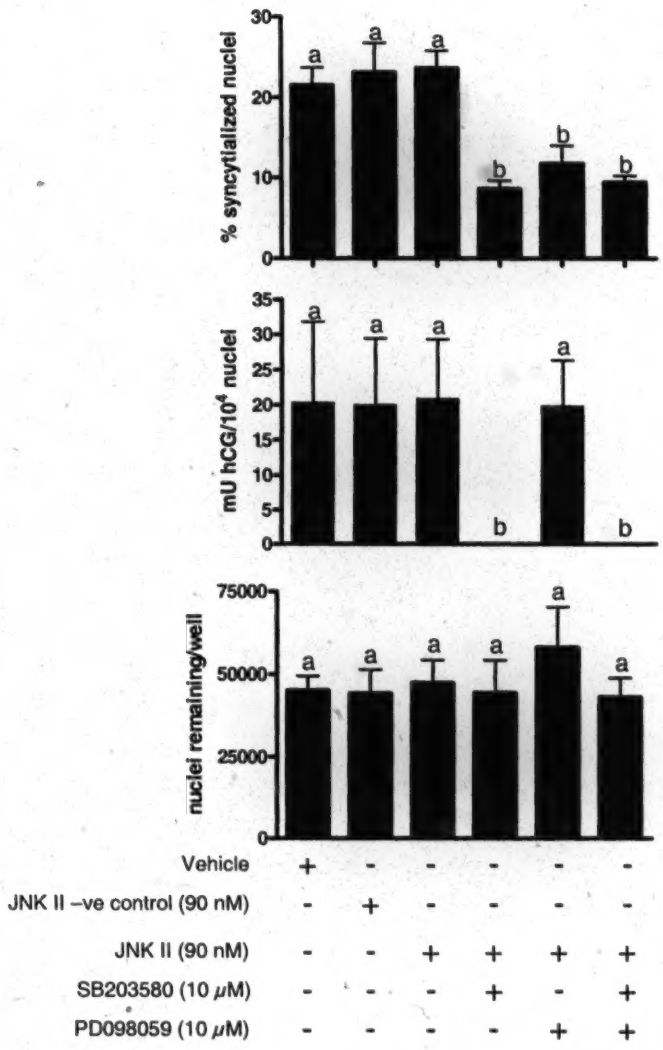


Figure 4.16. The effect of inhibition of JNK MAPK with JNK II Inhibitor on trophoblast differentiation (syncytialization and hCG production) and survival (nuclei remaining) in normal (n=4) trophoblast cultures. a) JNK inhibition alone did not alter trophoblast syncytialization, however in combination with ERK1/2 inhibition (PD098059) and p38 MAPK inhibition (SB203580), syncytialization was decreased. b) JNK inhibition alone did not effect hCG production in normal trophoblast cultures, but in combination with p38 MAPK inhibition, hCG production was decreased. c) JNK inhibition, either alone or in combination with ERK1/2 or p38 MAPK inhibition did not effect cell survival as reflected by unchanged numbers of nuclei remaining with each treatment. Values are presented as mean ± standard error of the mean and statistical significance determined by 1-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

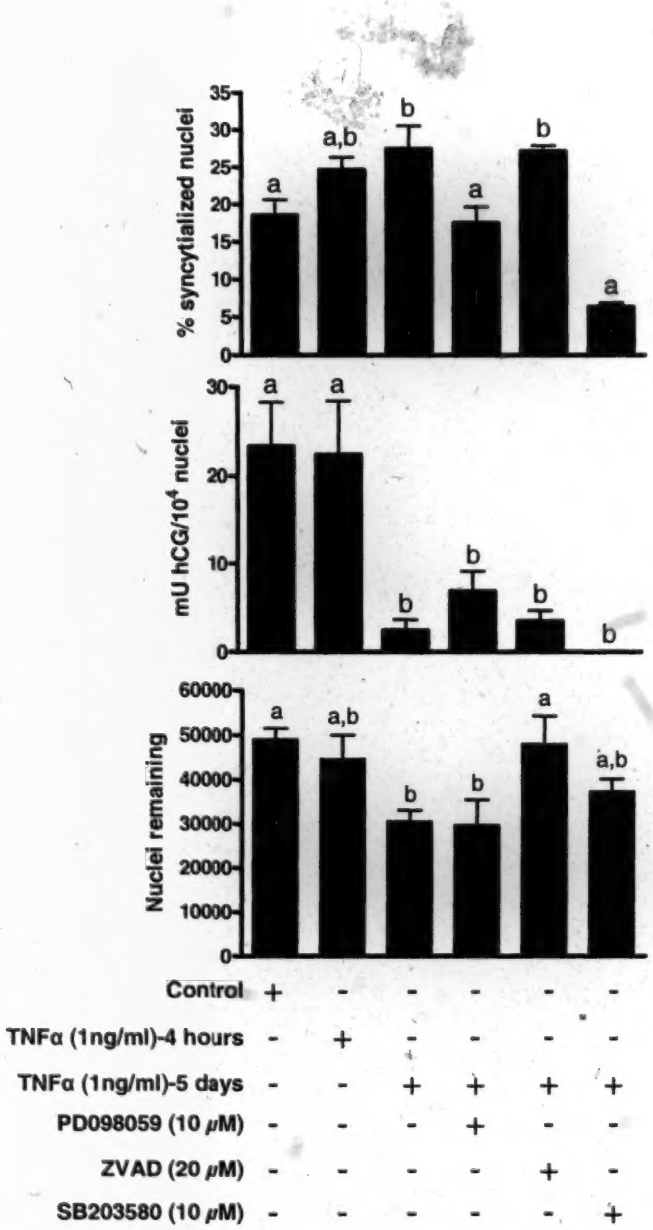


Figure 4.17. The effect of 1 ng/ml of TNFα on syncytialization (upper panel) and hCG production (lower panel) with and without inhibition of ERK1/2, p38 MAPK and caspase activity. 1 ng/ml of TNFα stimulates syncytialization in normal trophoblasts and is suppressed by inhibition of p38 MAPK (SB203580) and ERK1/2 (PD098059) but is not effected by caspase inhibition. TNFα decreases hCG production and is further decreased by p38 MAPK inhibition (SB203580). Although not significantly different from TNFα alone, inhibition of ERK1/2 slightly rescued TNFα suppressed hCG production (bottom panel). Values are presented as mean ± standard error of the mean and statistical significance determined by 1-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

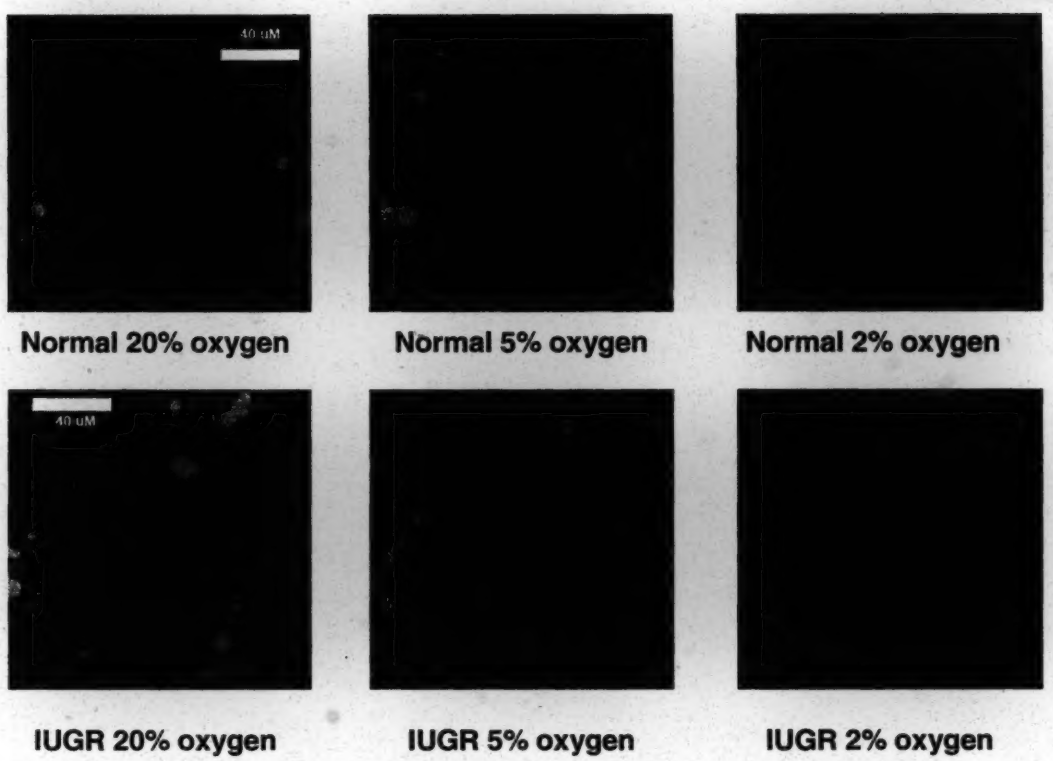


Figure 4.18. The effect of oxygen on desmoplakin defined syncytialization in normal and IUGR trophoblast cultures after 5 days. Oxygen tension does not alter spontaneous syncytialization in normal or IUGR trophoblasts. Desmoplakin (green) delinated areas with more than one nuclei (DAPI-blue) were considered syncytialized nuclei.

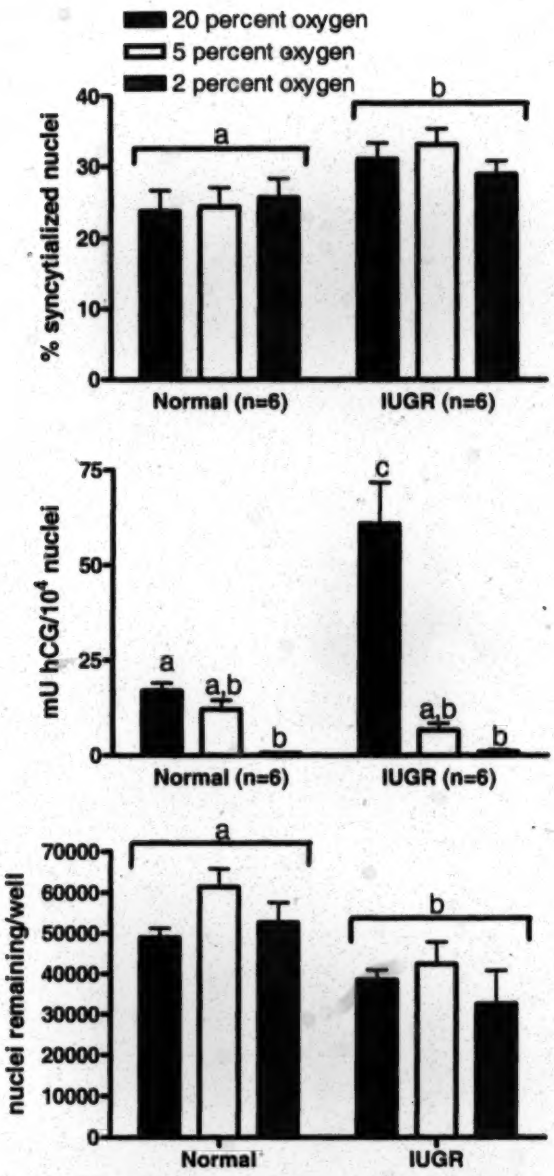


Figure 4.19. The effect of oxygen on trophoblast differentiation (syncytialization and hCG production) and survival in normal and IUGR after 5 days.
Syncytialization (upper panel) was unaffected by changes in oxygen tension. In contrast, hCG production (centre panel) was suppressed by low-normoxic (5%) and hypoxic (2%) oxygen tensions. The number of nuclei remaining (lower panel) was also unchanged by oxygen tension. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

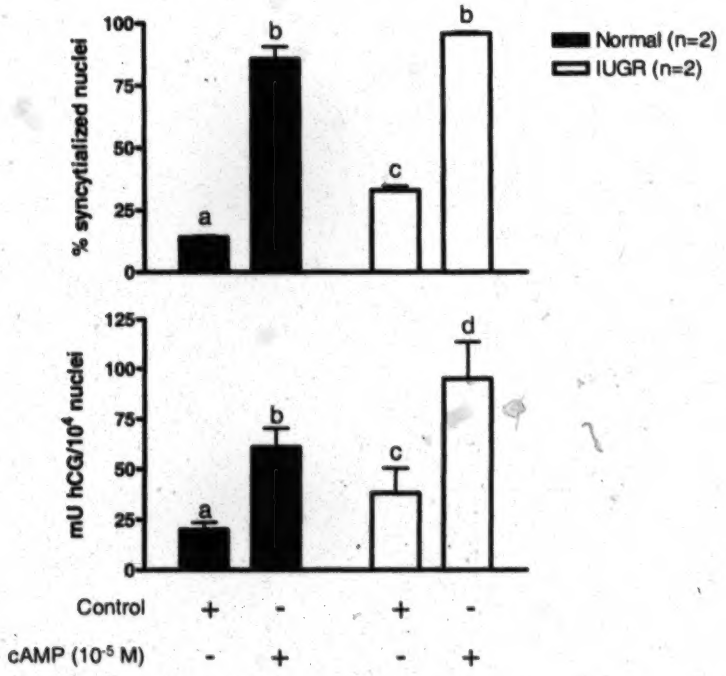


Figure 4.20. The effect of 8-bromo-cAMP on hCG production and syncytialization in normal and IUGR trophoblasts. 8-bromo-cAMP stimulates syncytialization (top panel) and hCG production (bottom panel) in both normal and IUGR trophoblasts. Values are presented as mean ± standard error of the mean and statistical significance determined by 2-way ANOVA with $p<0.05$ considered significant. Letters denote statistical significance, letters that are the same indicate no significant difference and those that are different indicate $p<0.05$.

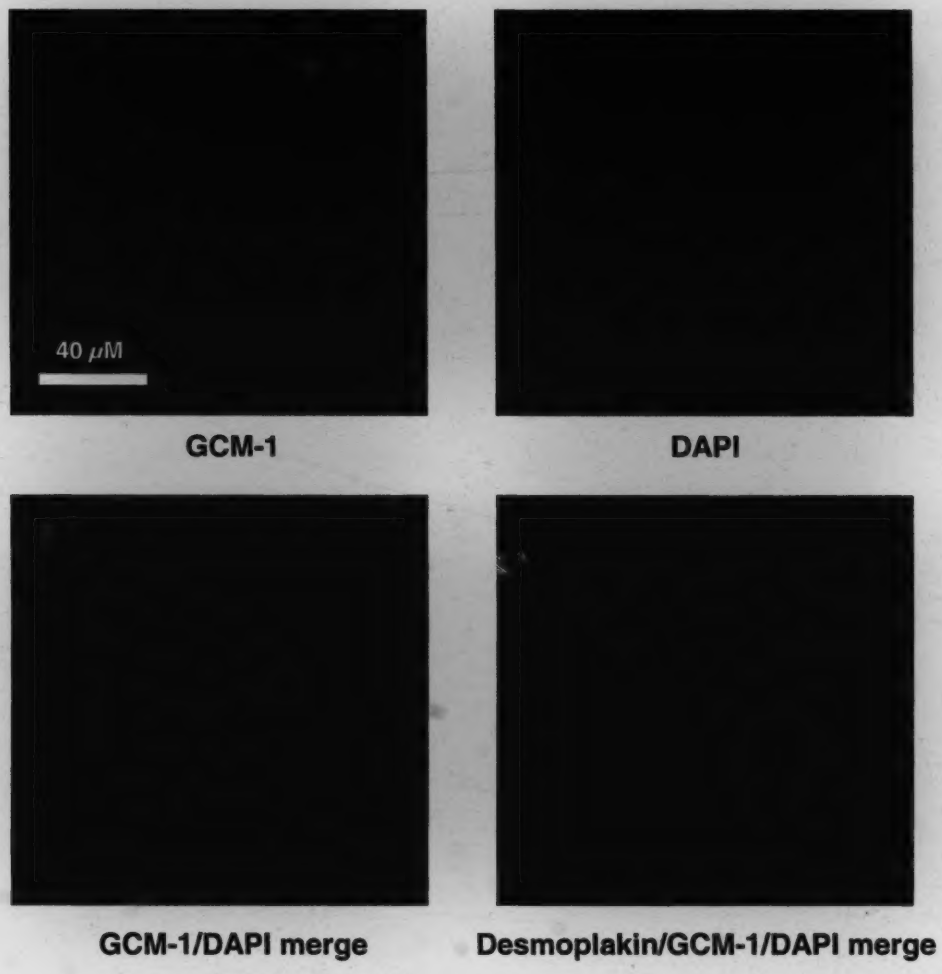


Figure 4.21. GCM-1 immunofluorescent staining in normal trophoblast 4 day culture. Top right panel: GCM-1 expression; Top left panel: DAPI stained nuclei; Bottom right panel: merged image of GCM-1 expression and DAPI stained nuclei; Bottom left panel: merged image of GCM-1 and desmoplakin expression and DAPI stained nuclei.

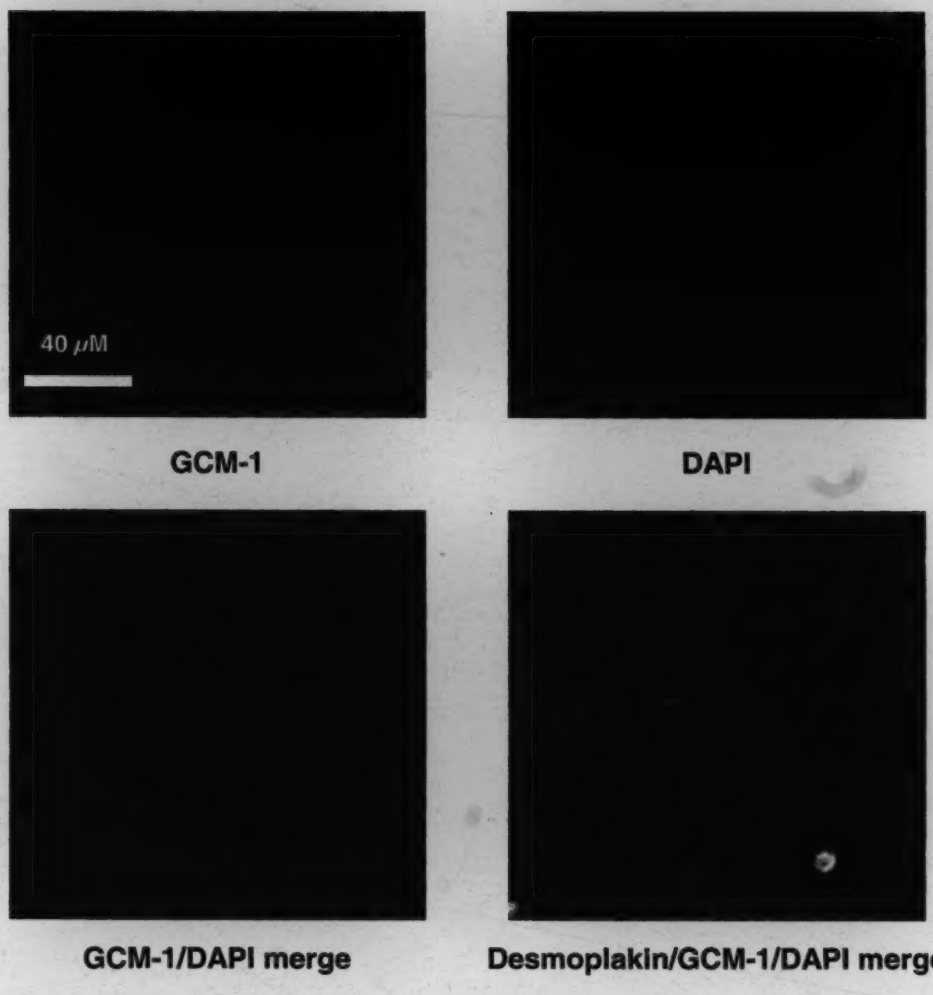


Figure 4.22. GCM-1 immunofluorescent staining after 4 days in normal trophoblast cultures induced with 8-bromo-cAMP (10^{-5} M). Top right panel: GCM-1 expression; Top left panel: DAPI stained nuclei; Bottom right panel: merged image of GCM-1 expression and DAPI stained nuclei; Bottom left panel: merged image of GCM-1 and desmoplakin expression and DAPI stained nuclei.

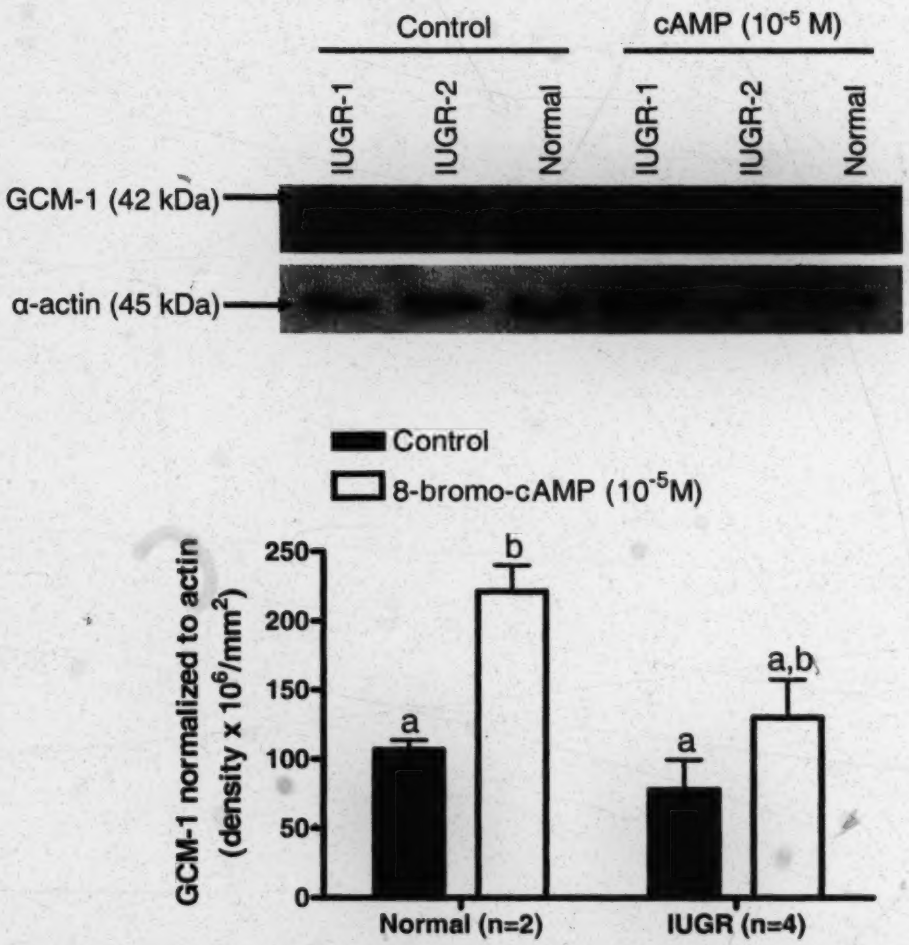


Figure 4.23. GCM-1 protein expression before and after 8-bromo-cAMP treatment on Day 3 of culture under standard conditions. In the upper panel, GCM-1 expression is shown in CT/ST mixed cultures from two different IUGR placentae and one normal placenta after 3 days in culture with and without 8-bromo-cAMP and the actin loading control. When GCM-1 expression is normalized to α-actin (bottom panel), there is an upregulation of GCM-1 expression upon cAMP treatment in both normal and IUGR trophoblast cultures when analyzed by 2-way ANOVA followed by Bonferroni post test. The letters indicate statistical significance, letters that are the same indicate no difference and those that are different show $p<0.05$. This experiment has been repeated twice with similar results, shown here is a representative of the two experiments.

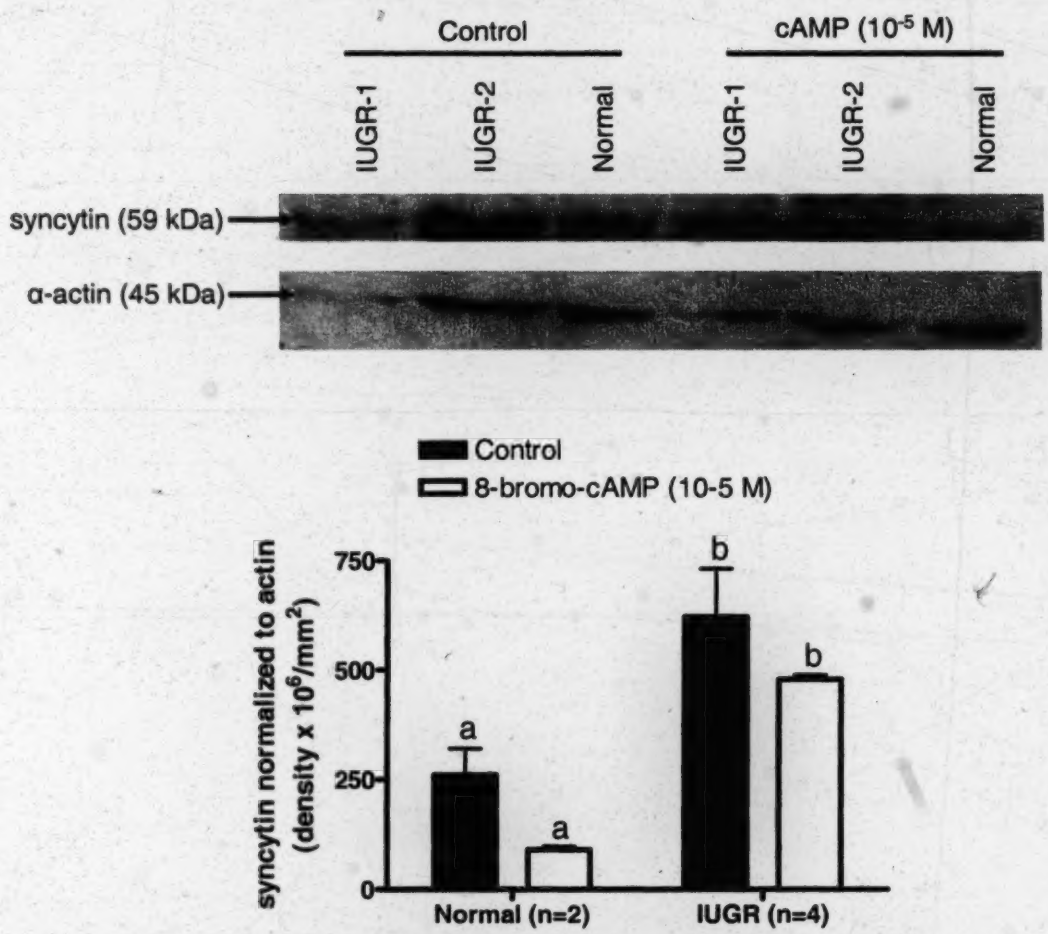


Figure 4.24. The effect of cAMP on syncytin protein expression in normal and IUGR trophoblast cultures. Normal (n=1) and IUGR (n=2) trophoblast cultures were cultured for 3 days under standard conditions with or without 8-bromo-cAMP. Syncytin expression density, when normalized to α-actin (bottom panel), was increased in IUGR trophoblasts compared to normal and is decreased by cAMP treatment in both normal and IUGR when compared by 2-way ANOVA followed by Bonferroni post test with $p<0.05$ considered statistically significant. The letters indicate statistical significance, the same being no different. This experiment has been repeated twice with similar results, shown here is a representative of the two experiments.

CHAPTER 5: DISCUSSION

5.1 Overview (references in introduction)

Trophoblast differentiation is a highly regulated process involving multiple interlinked signaling pathways. Throughout placental development, the coordinate regulation of CT proliferation, CT fusion with the overlying ST (trophoblast differentiation) and the maintenance of a functional barrier between the maternal and fetal circulation is essential to pregnancy as well as the proper development of the fetus. When inappropriate placental development occurs, the placenta is unable to maintain the adequate supply of nutrients to the developing fetus. This can result in compromised fetal growth and lead to the development of IUGR, a condition in which the fetus fails to reach its full optimal growth potential.

Despite decades of study, the etiology of IUGR remains unknown. IUGR is known to be associated with increased levels of TNFα in both maternal serum and amniotic fluid indicating an environment of inflammation. In addition, IUGR placentae have increased rates of apoptosis in both the CT and ST, indicating elevated apoptosis may contribute to the pathophysiology of this disease. I propose that in a trophoblast culture model of differentiation, elevated levels of the proinflammatory cytokine TNFα mediate increased trophoblast apoptosis and decreased trophoblast differentiation leading to placental insufficiency associated with IUGR.

In this study, I have investigated the signaling pathways by which TNFα influences trophoblast differentiation and apoptosis, the combination of which may lead to the placental dysfunction associated with IUGR.

5.2 Trophoblast Apoptosis and Cell Loss is Accelerated in IUGR

Previously, our lab has shown that IUGR CT cultures have increased rates of apoptosis compared to normal CT cultures (Kilani et al, submitted). Here I show that after 5 days in culture IUGR mixed CT/ST cultures also have elevated apoptosis compared to normal (**Figure 4.1**). In addition, there are fewer nuclei remaining in IUGR trophoblast cultures than in normal cultures after 5 days in culture in three different oxygen tensions which correspond to hyperoxia (18%), normoxia (5%) and hypoxia (2%)

within the *in vivo* placental environment (**Figure 4.19**). Addition of neutralizing antibody to TNFα decreased apoptosis in IUGR trophoblast cultures (**Figure 4.3**) as well as the number of nuclei lost over the culture period (**Figure 4.4**), indicating that either IUGR trophoblasts are more sensitive to the effects of endogenous TNFα or the IUGR trophoblast cultures are synthesizing more TNFα. This confirmed the first part of my hypothesis in that TNFα was contributing to the increased apoptosis in IUGR trophoblast cultures.

5.3 Trophoblast Differentiation is Elevated in IUGR Trophoblast Cultures

Although much is known about the regulation of trophoblast differentiation in primary villous CT populations isolated from normal placentae (7, 8, 19, 31, 36, 43, 47, 51, 52, 56, 65, 99, 101, 114, 124, 149, 171, 209), little is known about differentiation in IUGR villous CT populations as few groups in the world are able to both isolate pure populations of villous CT and collect IUGR placentae. I hypothesized that IUGR trophoblasts would have decreased differentiation compared to normal cultures and that this effect may be mediated by TNFα. In contrast to my hypothesis, I found that IUGR trophoblast cultures underwent more differentiation as assessed by increased syncytialization, and increased hCG and hPL production compared to normal trophoblast cultures (**Figure 4.2**).

IUGR is often associated with preeclampsia. However, in IUGR, the disease affects only the placenta and the developing fetus (198) whereas in IUGR/preeclampsia, described as a disease of the maternal vasculature (176), both the maternal and fetal systems are affected (48). A recent review shows that there are some important differences between IUGR and preeclampsia (162), thus I wanted to prevent further complicating the interpretation of my data by separating IUGR and IUGR-PE. Surprisingly, IUGR-PE trophoblast cultures behaved similarly to normal trophoblast cultures, having the same amounts of syncytialization and production of hCG and hPL. This could be due to the preelampsia-associated increase in expression of PL74, a TGFβ family member, which, in addition to promoting apoptosis, decreases trophoblast differentiation (syncytialization) *in vitro* (133). Although the similarity between IUGR-PE and normal trophoblast cultures was surprising, I decided to focus on further

exploring the pathways mediating elevated trophoblast differentiation in the IUGR group. All further experiments were to determine how IUGR trophoblast signaling was different from normal and how this signaling was contributing to increased differentiation.

5.4 Trophoblast Differentiation in IUGR is Mediated by TNFα

In cultures treated with neutralizing antibody to TNFα, not only was apoptosis decreased, I also found that both elevated syncytialization and hCG production was blocked (**Figure 4.4**). This indicated that TNFα was acting through some signaling intermediate to stimulate accelerated trophoblast differentiation in IUGR. Previous work in our lab showed that EGF activation of p38 MAPK signaling is required for both hCG production and syncytialization (101). This lead to the hypothesis that TNFα was signaling through p38 MAPK to stimulate hCG production and syncytialization in IUGR trophoblast cultures.

5.5 TNFα Stimulates Caspase Cleavage and MAPK Phosphorylation

As the majority of TNFα signaling pathways remained unknown in the primary villous trophoblast, I first determined which signaling pathways were activated by TNFα. I found that addition of TNFα to normal trophoblast cultures resulted in strong phosphorylation of p38 MAPK (**Figure 4.5**), ERK1/2 (**Figure 4.6**) and JNK (**Figure 4.7**) in addition to caspase 3 cleavage (**Figure 4.8-d**). Contrary to TNFα signaling in epithelial cells (49), I was unable to detect significant caspase 8 cleavage at any of the three time points (6, 12 or 18 hours) after TNFα addition (**Figure 4.8-a,b,c**). This could indicate either that in the primary villous trophoblast, TNFα does not stimulate caspase 8 cleavage and instead acts through a caspase 8 independent mechanism to induce apoptosis or that the small amount of apparent caspase 8 cleavage occurred prior to the time points I chose.

Caspase 8 appears to be cleaved as indicated by the presence of low molecular weight bands (**Figure 4.8-a,b,c**). However, the appearance of the cleavage products was very low relative to the procaspase form and was not stimulated by the presence of TNFα. In addition, the appearance of the cleavage products was not inhibited by the presence of ZVAD-fmk. However, I found that ZVAD was able to block caspase 3 cleavage (**Figure**

4.8-d). Previous work has shown that ZVAD is a pan-caspase inhibitor that completely blocks caspase 8 cleavage at 20 μM (150), which is the concentration used in this study. This means that either the cleavage bands seen on the western were not specific to caspase 8 or that caspase-8 cleavage happens prior to the addition of ZVAD and the cleavage products are stable. I believe that villous trophoblast cultures are refractory to caspase-8 activation. This may be due to an upregulation in anti-apoptotic proteins (such as FLIPs, Bcl-2 and Mcl-1) that occurs prior to or during isolation which directly prevent caspase-8 cleavage OR an alternative signaling pathway for TNFα through TNFR1 which does not involve caspase 8. It is a future aim of our group to determine if caspase-8 is indeed activated and the mechanism by which this occurs. Some studies suggest that CD95 (or Fas) is required for caspase-8 activation (150). In addition, other authors have shown that villous trophoblast (both CT and ST) express FLIP proteins (106), which act directly to inhibit the activation of caspase 8. Others have shown that trophoblasts are unable to undergo apoptosis stimulated through Fas due to the presence of XIAP proteins which prevent activation of caspase 9 and caspase 3 (196). Taking these results into consideration, it seems likely that the overexpression of an antiapoptotic protein such as FLIP in addition to the lack of apoptotic stimuli able to signal through Fas lends the trophoblast largely refractory to caspase 8 activation. Indeed, our lab has shown directly that our villous CT population is not able to undergo CD95/Fas mediated apoptosis (168). However, lack of caspase 8 signaling due to the presence of excess FLIP remains to be proven in our system.

5.6 Elevated Differentiation in IUGR Trophoblast Cultures Requires Activity of ERK1/2 and p38 MAPKα

As TNFα was able to stimulate p38 MAPK phosphorylation and our lab has previously shown p38 MAPK activity to be required for both syncytialization and hCG production (101), I wanted to determine if inhibition of p38 MAPK could decrease the elevated differentiation seen in IUGR trophoblast cultures. Other researchers have shown that in primary villous trophoblasts isolated by the Kliman method (cultures reaching maximal differentiation in 4 days and viability limited to roughly 6 days) both p38 MAPK and ERK1/2 are involved in the differentiation process (51). As I showed that

TNFα was also able to stimulated ERK1/2 phosphorylation, I wanted to determine if ERK1/2 was responsible for mediating increased differentiation seen in IUGR trophoblast cultures.

Inhibition of the p38 MAPKα isoform with 10 μM of SB203580 resulted in a significant reduction in both syncytialization and hCG production in both IUGR and normal trophoblast cultures (**Figure 4.13**). In contrast, inhibition of ERK1/2 with 10 μM of PD098059 only decreased the two differentiation markers (syncytialization and hCG production) in IUGR trophoblast cultures and had no effect on normal cultures. This indicates that increased signaling through ERK1/2 in IUGR can mediate both elevated syncytialization and hCG production. Providing supporting evidence for this has become a future goal of our lab.

5.7 Exogenous TNFα has Differential Effects on Trophoblast Differentiation

Some of the previous work characterizing the effect of TNFα on trophoblast function was carried out using two choriocarcinoma derived cell lines (5), placental explants (46) and primary villous trophoblasts isolated by the Kliman method (131). In the two choriocarcinoma cell lines, the non-fusogenic JEG-3 cells and fusogenic BeWo cells were exposed to TNFα, resulting in increased apoptosis in both cell types but a loss in viability in only the BeWo cell line (5). The loss in viability (as assessed by a decrease in the mitochondrial activity) indicates a potential role for necrosis in these cells in response to TNFα. As both cell lines were derived from cancer cells, it is highly problematic to extrapolate these findings to primary human trophoblast cultures. In explants and primary villous trophoblast cultures, TNFα was found to decrease hCG production (46, 131), which is in agreement with my findings that hCG production is decreased upon TNFα exposure in a dose dependent manner (**Figure 4.13**). In contrast to the findings in a recent paper in which TNFα decreased syncytialization(131), when I exposed normal trophoblast cultures to increasing doses of TNFα, I found TNFα stimulates syncytialization (**Figure 4.12**). This increase in syncytialization of normal trophoblast cultures can be prevented by inhibition of ERK1/2 phosphorylation with PD098059 (**Figure 4.18**). Differences in the method of trophoblast isolation may explain the differential response to TNFα between these two populations as our trophoblasts

differentiate more slowly and are able to survive for longer culture periods, thus more closely mimic the *in vivo* life span of the trophoblast which ranges from 3 to 4 weeks (93).

5.8 The Role of TNFα in Trophoblast Turnover

The presence of TNFα in the normal human placenta indicates it may play a role in placental development (155). Apoptosis is a natural process in the placenta itself and it is thought to play a role in trophoblast turnover as indicated by a small percentage of TUNEL positive nuclei present in both the CT and ST layers of normal placentae (197). Trophoblast turnover has multiple components, beginning with proliferation of the CT stem cells, followed by fusion of daughter CT cells into the overlying syncytium, progression towards apoptotic cell death and culminating in the extrusion of groups of mainly (90%) apoptotic nuclei (2) surrounded by synycytial plasma membrane into the maternal circulation as syncytial knots (93). In this study, I show that TNFα mediates the increased apoptosis (**Figure 4.4**) and cell loss (**Figure 4.5-c**) seen in IUGR trophoblast cultures. I also show that TNFα signaling through ERK1/2 and to a lesser extent p38 MAPK results in increased differentiation (both syncytialization and hCG production) in IUGR trophoblasts (**Figure 4.5, 4.14, and 4.18**). I propose that the combination of both increased apoptosis and increased differentiation could lead to a reduction in the number of CT available for maintenance of the ST, resulting in either breaks in the ST barrier leading to placental villitis or increased trophoblast turnover characterized by decreased villous area and increased shedding of syncytial knots. I also show that exogenous TNFα can both stimulate and inhibit trophoblast differentiation markers by stimulating syncytialization (**Figure 4.11-a**) and inhibiting hCG production (**Figure 4.12**) when TNFα is present over long periods of time. It could be that the presence of TNFα at plating alone stimulates ERK1/2 and p38 MAPK signaling to lead to increased syncytialization and stimulates hCG production by day 5. However, when TNFα is present for longer periods of time, it is able to activate the caspase cascade and lead to cell loss, possibly resulting in the death of those cells which are responsible for hCG production. I show this indirectly in **Figure 4.17** in that exogenous TNFα present during plating slightly stimulates syncytialization compared to untreated controls but it also does

not inhibit hCG production. In contrast, TNFα (at the same dose) present during the entire culture period leads to increased cell loss, increased syncytialization and decreased hCG production. This could have implications for IUGR in that the long term presence of exogenous TNFα in the villous placenta leads to increased apoptosis, increased syncytialization and decreased functional differentiation, the combination of which could contribute to the pathophysiology of this disease. Determination of whether TNFα is actively killing hCG producing cells is a future goal of our group. **Figure 5.1** shows the proposed signaling pathways activated by TNFα which I have shown to be involved in and required for trophoblast differentiation.

5.9 Differential Effects of Exogenous and Endogenous TNFα

The actions of endogenous TNFα are directly blocked by neutralizing antibody to TNFα, reflecting the effects of the amount of TNFα produced directly by the cultures. The fact that neutralizing antibody to TNFα suppressed bot elevated syncytialization and hCG production in IUGR trophoblast cultures (**Figure 4.4**) suggests that these cultures are releasing a larger basal amount of TNFα than normal cultures. Production and release of TNFα by trophoblasts occurs continuously and in small amounts, likely less than 0.01 ng/ml, which allows for continuous low level stimulation of the signaling pathways activated by TNFα. In this way, endogenous TNFα action is much different than that following addition of exogenous TNFα. Addition of exogenous TNFα allows for maximal but transient stimulation of TNFα signaling pathways, as seen by previous work in our lab which shows that 24 hours after addition of TNFα (10 ng/ml), all of the TNFα has been used by the trophoblast cultures (L. Guilbert and R. Kilani). Thus, addition of exogenous TNFα is a phasic response, allowing for stimulation in bursts after addition of TNFα followed by a lack of TNFα stimulated signaling. This is much different from a low level of constant stimulation such as that due to endogenous TNFα. This could contribute to the differential response to exogenous as compared to endogenous TNFα. Blocking endogenous TNFα decreases syncytialization and hCG production, which suggests that TNFα is able to stimulate both processes (**Figure 4.4**). In contrast, addition of exogenous TNFα to normal and IUGR trophoblast cultures had differential effects on syncytialization (which was stimulated in normal trophoblasts but not in IUGR-**Figure**

4.11-a) and hCG production (in which exogenous TNFα dose dependently decreased hCG production in both normal and IUGR-**Figure 4.12**). Interestingly, when I compared total nuclei number in ST versus CT (instead of % syncytialization), I found that normal ST numbers were unaffected by dose of TNFα, but the highest dose of TNFα significantly decreased the number of ST nuclei in IUGR trophoblast cultures (**Figure 4.11-b**). In contrast, CT nuclei number in both normal and IUGR was dose dependently decreased by TNFα (**Figure 4.11-b**). Thus, exogenous TNFα induces a decrease in CT nuclei in both normal and IUGR but only decreases ST nuclei in IUGR. This may contribute to the significant increase in the percent syncytialization in normal trophoblast cultures seen at 1 and 10 ng/ml of TNFα that is not seen in IUGR.

TNFα signals through both TNFR1 and TNFR2, cell surface receptors which are both expressed by the trophoblast (123). In IUGR, there is an increase in TNFR1 mRNA (Kilani *et al*, submitted). However, it is unknown if TNFR2 expression is altered by IUGR. TNFα is able to bind and signal through both TNFR1 and TNFR2, but there is evidence to show that TNFR1 has a higher affinity for TNFα and signaling through TNFR1 is different from signaling through TNFR2, although this depends on cell type and origin (32, 140). TNFR1 signaling is able to activate the MAPK cascade and caspases directly whichas TNFR2 signaling can aid in TNFR1 activation of apoptosis signaling independent of direct caspase association with the receptor as TNFR2 does not have a death domain and is not directly bound to either caspase 8 or 2 (140). Characterization of TNFR1 and R2 expression in the villous trophoblast revealed that although both receptors are expressed, TNFα signals through TNFR1 to induce cytotoxicity (214). Future work in our lab may focus on delineating the contributions of TNFR1 and TNFR2 signaling within the trophoblast using neutralizing antibodies for each receptor to see if TNFR2 signaling is more involved in the differentiation pathway.

5.10 Interleukin-1β Is Not Involved in Trophoblast Differentiation *In Vitro*

Previous studies suggested that IL-1β was involved in trophoblast differentiation through an IL-6/NFκB dependent signaling pathway which was able to stimulate hCG production (109, 113, 200). As IL-1β is a pro-inflammatory cytokine and hCG

production was elevated in IUGR, I hypothesized that IL-1β may play a role. However, addition of excess neutralizing antibody to IL-1β affected neither marker of trophoblast differentiation in that both syncytialization and hCG production remained unchanged from the isotype control (**Figure 4.4**). Previous IL-1β signaling work was done using choriocarcinoma cell lines (BeWo (200) and JAR (97)), both of neoplastic origin and therefore may not display normal trophoblast signaling. Using JAR cells, the authors found that both TNFα and IL-1β, signaling through ERK1/2 MAPK, stimulated trophoblastic hCG production (97). It would be important to confirm that I had neutralized all IL-1β activity by adding IL-1β in addition to neutralizing antibody. Additionally, although endogenous IL-1β did not seem to be involved in the regulation of trophoblast differentiation, application of exogenous IL-1β may stimulate trophoblast differentiation. Future work in our group will focus on whether exogenous IL-1β is able to induce differentiation and the mechanisms involved.

5.11 The Caspase Cascade is Not Involved in Trophoblast Differentiation *In Vitro*

Although a large number of recent reviews have focused on the involvement of the apoptosis cascade in trophoblast differentiation (13, 34, 81, 90, 92-94, 144, 156, 157, 197), there is still limited evidence for its direct involvement in this complex process *in vitro*. Experiments using primary trophoblast cultures isolated by the Kliman method demonstrated that caspases are more active in CT cultures than in ST cultures (215), indicating CT may be more sensitive to apoptotic stimuli than ST. Other work looking at the expression pattern of inhibitors of apoptotis (IAP) proteins (which can inhibit the activity of caspases) demonstrated that trophoblast lineages (villous CT versus villous ST versus extravillous trophoblast) display different patterns of IAP expression indicating differential regulation of apoptosis during trophoblast differentiation (107). Huppertz and collegues have shown increased expression of anti-apoptotic proteins Bcl-2 and Mcl-1 in the ST compared to the CT as well as focal areas of apoptotic nuclei representing areas of syncytial knot formation in formalin-fixed tissue sections of placental explants (89). They have also shown that caspase 8 activation is required for trophoblast differentiation in the placental explant model (16), however this work is less convincing in that cell signaling within a mixed cell population, such as that found within the placental explant,

does not directly prove that caspase 8 activation within the villous trophoblast is the necessary event that mediates trophoblast differentiation.

In contrast to the results presented by the Huppertz group in the placental explant model, I found that trophoblast differentiation in highly purified villous CT cultures was not dependent on caspase cleavage as addition of the pan-caspase inhibitor ZVAD-fmk did not effect syncytialization or hCG production (**Figure 4.15**) in either normal or IUGR trophoblast cultures. My results are supported by other members of our group (Larry Guilbert and Bonnie Lowen) who have found the ZVAD-fmk does not inhibit villous explant production of hCG.

5.12 IUGR Trophoblast Cultures are Responsive to cAMP Induced Differentiation

As exogenous TNFα did not stimulate syncytialization in IUGR trophoblast cultures (**Figure 4.11-a**), I was concerned that IUGR trophoblast cultures were unable to undergo differentiation in vitro. To test this hypothesis, I used 8-bromo-cAMP to stimulate trophoblast differentiation in both normal and IUGR trophoblast cultures (**Figure 4.20**). I found that addition of 10^{-5} M of the cAMP analogue 8-bromo-cAMP significantly increased both hCG production and syncytialization in IUGR trophoblast cultures. Interestingly, there was significantly more hCG produced in IUGR than normal trophoblast cultures after cAMP treatment. Thus, even though IUGR trophoblast cultures already had an elevated level of hCG production, they could be further stimulated to produce even more hCG with the addition of 8-bromo-cAMP, which led to even more production than normal trophoblast cultures.

In addition to syncytialization and hCG production as markers of cAMP induced differentiation, I also examined GCM-1 and syncytin expression. Previous studies have shown that GCM-1, a transcription factor expressed in the placenta, is upregulated in CT in response to cAMP (37) and that GCM-1 expression is specific to the villous CT which are destined for ST fusion (8). I first showed that GCM-1 expression via immunofluorescence is localized to both the cytoplasm and the nuclei of normal CT/ST (uninduced Day 3) cultures (**Figure 4.21**), but upon addition of 8-bromo-cAMP there was a shift in GCM-1 expression to mainly nuclear localization (**Figure 4.22**) in normal ST (80% syncytialized with cAMP on Day 3) cultures. Once I had determined that the

villous CT population expressed GCM-1, I wanted to determine if GCM-1 expression was different in normal and IUGR trophoblast cultures. I found that although the two differentiation markers (syncytialization and hCG production) were consistently higher in IUGR trophoblast cultures compared to normal, there was not a significant trend in increased GCM-1 expression in IUGR trophoblast cultures compared to normal cultures on day 3 (**Figure 4.23**). However, cAMP treatment did significantly upregulate GCM-1 protein expression in both normal and IUGR trophoblast cultures (**Figure 4.23**). In contrast, expression of syncytin appeared to be consistently higher in IUGR trophoblast cultures compared to normal and was not increased by cAMP treatment in either group (**Figure 4.24**). Syncytin-1 expression is not specific to the villous trophoblast as it is also expressed in the extravillous trophoblast (159) but is not expressed in non-placental tissues due to methylation of the long term repeat region which prevents transcription (148). However, this suppression may be aberrant in some cancer cells (15). Future work in our lab will focus on examining the cellular expression pattern of syncytin and GCM-1.

5.13 The Role of Oxygen in Trophoblast Differentiation

The majority of *in vitro* studies focusing on trophoblast differentiation have been carried out under standard culture conditions in which the oxygen tension is ~138 mmHg or 18% oxygen. Unfortunately, these conditions of supraphysiologic oxygen (hyperoxia) compared to normoxic conditions (between 5 and 8% oxygen) within the placenta *in vivo* may lead to altered signaling which may confound interpretation of the data. Previous studies have shown that oxygen itself can modulate trophoblast turnover [proliferation (72), differentiation (31), and death (46, 115, 141)]. As such, I wanted to determine if oxygen tension would affect the elevated differentiation seen in IUGR. I cultured normal and IUGR trophoblasts at 2% (hypoxic conditions), 5% (low normoxic conditions) and 18% (hyperoxia) for 5 days. I found that oxygen tension had no effect on syncytialization (**Figure 4.19**) and IUGR syncytialization remained significantly elevated compared to normal trophoblast cultures. However, hCG production over the first 5 days in culture was dramatically suppressed by decreasing oxygen tensions (**Figure 4.19**) in both normal and IUGR trophoblast cultures. Although hCG production was inhibited by

low oxygen, preliminary experiments that I preformed using placental explants cultured at 5% oxygen showed that the peak of hCG production was between Day 7 and Day 9 (data not shown). It could be that hCG production is merely delayed at normoxic conditions for the trophoblast and that slower production of hCG more closely represents the *in vivo* situation in which the life span of the trophoblast is extended and the differentiation process is slower.

5.14 Morphologic and Functional Trophoblast Differentiation are Separate Processes

Throughout this study, I have shown evidence for the separation of two markers of trophoblast differentiation: syncytialization (morphologic) and hCG production (functional). This is shown by the differential effects of exogenous TNFα on syncytialization (in which TNFα stimulated syncytialization in a dose dependent manner) and hCG production (in which TNFα inhibited hCG production in a dose dependent manner). This is also proven by the effect of oxygen tension on syncytialization: normoxic and hypoxic conditions do not alter syncytialization but inhibit hCG production during 5 day culture periods. However, the opposite effect is seen when inhibition of both ERK1/2 and JNK MAPKs occurs in normal trophoblast cultures: syncytialization is decreased in cultures treated with ERK1/2 and JNK inhibitors but hCG production remains unchanged (**Figure 4.16**). Syncytialization itself can occur even though hCG production is inhibited. This goes against previous work that shows hCG production is a requirement for morphologic differentiation of the trophoblast (209). The findings presented in this thesis demonstrate that although much is known about trophoblast differentiation *in vitro* and show that morphologic and functional differentiation are two separate processes, this may not be the case in the *in vivo* environment of the placenta.

5.15 IUGR and TNFα: Cause or Result?

A pro-inflammatory environment exists within the IUGR placenta, as suggested by increased levels of TNFα and apoptotic nuclei. Inadequate placental development, leading to decreased nutrient delivery to the developing fetus, has also been implicated as a cause of IUGR. The following question arises when considering the role TNFα plays in

the development of IUGR: Is the maternal immune system responsible for decreased placental development and inflammation found in IUGR placentae (as in the case of the antiphospholipid syndrome in which the maternal immune system does not adapt to the semi-allogeneic fetus) OR are the fetal placental cells themselves producing more of the proinflammatory cytokine which consequently leads to IUGR? In the first situation, inadequate tolerance by the maternal immune system could lead the initiation of an inflammatory response, resulting in an increase in TNFα, leading to placental villitis and subsequent loss of the ST layer. If this occurred in focal areas, it could influence the entire syncytium at some later point in pregnancy, resulting in increased TNFα release by the ST. In the second situation, something fundamentally flawed in the regulation of TNFα biosynthesis could lead to the abberant elevated production of TNFα by the fetal trophoblast. Increased TNFα could then act to stimulate both elevated apoptosis (through a caspase 3 dependent pathway) and elevated differentiation (through the p38 MAPKα and ERK1/2 dependent pathways), ultimately culminating in increased trophoblast turnover. Accelerated turnover of the trophoblast then leads to decreased placental function as accelerated ST fusion and shedding leads to decreased surface area for exchange between the maternal and fetal systems.

5.16 Does mode of delivery and smoking affect villous trophoblast cultures?

This study addressed the question of whether IUGR and IUGR-PE villous trophoblast cultures behaved differently from normal trophoblast cultures *in vitro*. Once the placentae were collected by the lab, the procedures for isolating the villous CT population were identical, using a well established protocol and the same reagents. These factors are all things that can be (and were) controlled for in this study. However, due to the unavoidable nature of human variation, there were some differences between the normal, IUGR and IUGR-PE groups that were not controlled for. Firstly, our current lab protocol involves collection of normal placentae from mothers who deliver at a convenient predictable time (in the morning) which unfortunately involves collection of placentae delivered solely by ceasarian section. In contrast, IUGR and IUGR-PE placentae were delivered by both ceasarian and vaginal delivery. As such, this was one

uncontrolled for variable that may effect the villous CT population that we isolated. During vaginal delivery, contractions impede blood flow to the placenta and it has been suggested that this may cause focal apoptosis of the trophoblast (208). However, when I retrospectively separated the IUGR vaginal delivered CT from the IUGR CT delivered via caesarian section there was no significant difference in hCG production, syncytialization, apoptosis or response to any of the treatments. This suggests that the mode of delivery does not significantly impact the villous CT population that we isolate and does not alter the markers of trophoblast differentiation assessed in this study. In the future, it would be beneficial to strengthen the findings found in this work by collecting normal placentae delivered vaginally. Secondly, maternal age was not controlled for in this study, with a significant difference between normal and IUGR and IUGR-PE. Again, some evidence in the literature suggests within placental sections from normal pregnancies there is increased apoptosis and increased proliferation with increasing maternal age (208). Thirdly, maternal smoking was not controlled for in this study. Maternal smoking within all groups was self-reported and there is some evidence in the literature to suggest that we may have un-reported smokers within our normal placental group (in Manitoba 26.2% of non-Aboriginal and 61.2% of Aboriginal women smoked during pregnancy (80)). However, there were significantly higher numbers of mothers who smoked in the IUGR group (7 of 14) compared to the normal (1 of 14) or IUGR-PE (1 of 3) group (**Table 4.1**). I was concerned about this and omitted the IUGR placentae from smoking mothers from analysis and found the difference between all factors measured in normal and IUGR trophoblast cultures remained significant. Despite these confounding factors, I feel that my data does accurately depict a difference between cells isolated from the IUGR environment and those isolated from a normal pregnancy. However, in future work, our lab will aim to more closely match maternal age, maternal smoking and mode of delivery in order to control for these potentially confounding factors.

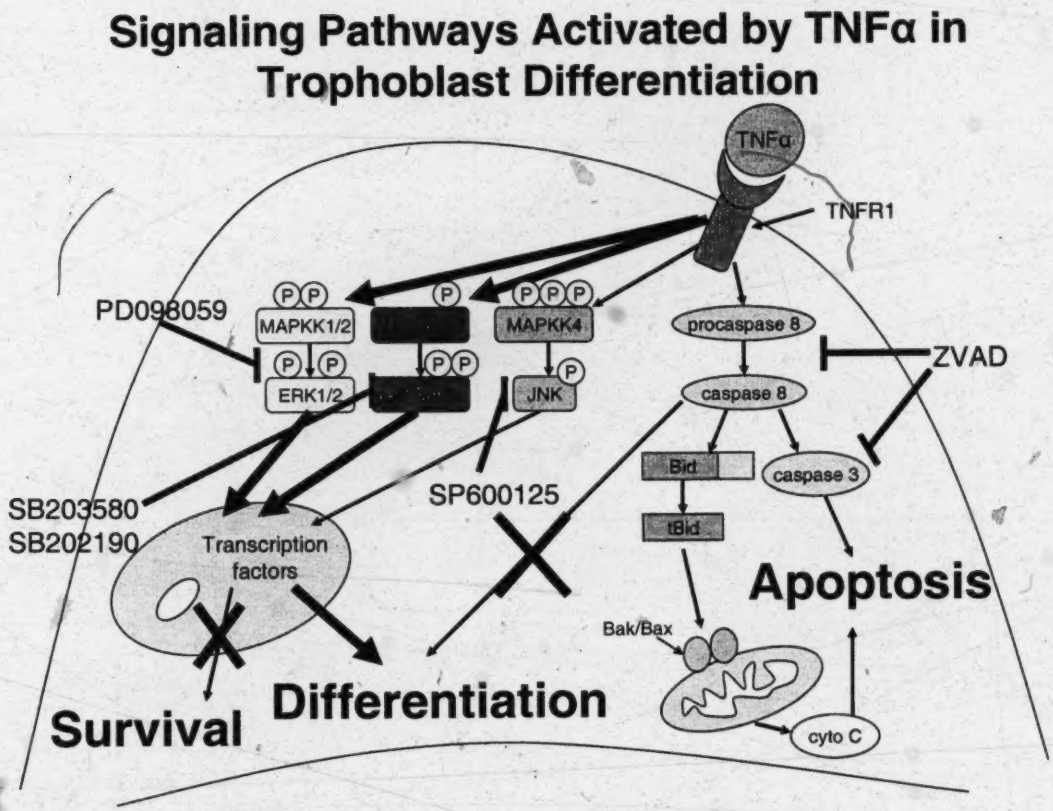


Figure 5.1 Signaling pathways activated by TNFα to stimulate trophoblast differentiation. TNFα binding to TNFR1 activates the phosphorylation of ERK1/2 and p38 MAPK. ERK1/2 phosphorylation stimulates elevated differentiation seen in IUGR. p38 MAPK phosphorylation is required for both hCG production and syncytialization.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 CONCLUSIONS

Despite decades of study, the etiology of IUGR remains unknown. IUGR is known to be associated with increased levels of TNFα in both maternal serum and amniotic fluid indicating an environment of inflammation. In addition, IUGR placentae have increased rates of apoptosis in both the CT and ST, indicating elevated apoptosis may contribute to the pathophysiology of this disease. I propose that elevated levels of the proinflammatory cytokine TNFα mediate increased trophoblast apoptosis and increased trophoblast differentiation leading to placental insufficiency associated with IUGR (**Figure 6.1**). In this study, I show that TNFα sensitivity and/or production are increased in IUGR trophoblast cultures. This increase leads to both increased apoptosis and increased differentiation (both syncytialization and hCG production). TNFα mediates apoptosis through a caspase-3 dependent mechanism, which is blocked by addition of neutralizing antibody to TNFα and by the pan-caspase inhibitor ZVAD. TNFα, signaling through ERK1/2 and p38 MAPKα, mediates increased syncytialization and hCG production seen in IUGR trophoblast cultures. Increased apoptosis and differentiation suggests that trophoblast turnover is accelerated in IUGR, leading to decreased placental function.

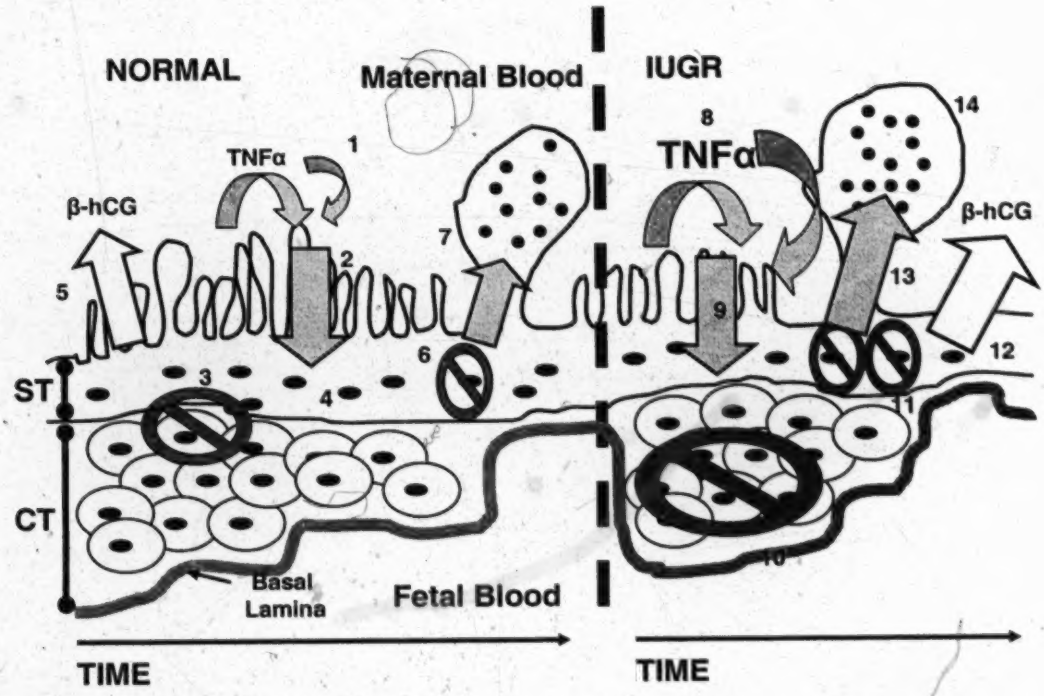


Figure 6.1. Overall schematic of TNFα effects in both normal and IUGR villous placentae.
In the normal placenta: **1**-TNFα (endogenously produced from the ST as well as exogenous from the maternal system) is present in low amounts in the normal placental envirnonment. **2**-TNFα signals through caspase cascade and p38/ERK/JNK MAPKs. **3**-Caspase signaling induces apoptosis in some CT cells. **4**-p38/ERK activation leads to minimal stimulation of CT fusion and JNK activation leads to survival. **5**-hCG production is unaltered or minimally stimulated by TNFα. **6**-ST apoptosis is induced to a small degree leading to **7**-The shedding of apoptotic nuclei in syncytial knots.

In the IUGR placenta: **8**-Elevated TNFα (from either endogenous ST production or exogenous maternal contributions) is present in IUGR placentae. **9**-TNFα signals strongly through the caspase cascade and ERK1/2 and p38 MAPK leading to **10**-The induction of increased CT apoptosis and CT fusion to form the ST. **11**-Increased TNFα signaling through caspases stimulates increased ST apoptosis and stimulation of ERK1/2 and p38 MAPK stimulates differentiation. **12**-Increased hCG production occurs due to elevated ERK1/2 and p38 signaling. **13**-Increased numbers of apopotitic nuclei enter syncytial knots. **14**-Increased shedding of syncytial knot debris occurs. In addition, the life span of the trophoblast is drastically decreased compared to the life span within the normal placenta, leading to increased trophoblast turnover.

6.2 FUTURE DIRECTIONS

As TNFα is able to stimulate trophoblast differentiation in a dose and time dependent fashion and GCM-1 is a temporally regulated transcription factor, it would be interesting to examine whether TNFα was able to induce GCM-1 expression. Also, as GCM-1 has been shown to modulate syncytin expression (211), the role of TNFα in syncytin expression would also be a novel pathway to examine.

As the preliminary experiments I carried out at hypoxic and normoxic oxygen tensions showed that trophoblast syncytialization was unaffected but hCG production was suppressed, further experiments involving hCG production over an extended culture period at normoxic (~5%) oxygen tensions would be valuable. This would be important to explore in both normal and IUGR trophoblast cultures, as the difference between the two populations is so striking at supraphysiologic oxygen tensions. It would be important to determine if elevated hCG production is actually a true marker of functional trophoblast differentiation or merely a stress response to elevated oxygen tensions. The life cycle of the trophoblast is estimated to be between 3 and 4 weeks (93). As such, the production of hCG within 3 days and peak at 5 days may be very far from the *in vivo* situation and may more closely resemble what I believe to be a slower rise in hCG production such as that that occurs at 5% oxygen.

In this work, I also showed that the combination of ERK1/2 and JNK inhibition leads to decreased syncytialization but does not effect hCG production. It would be valuable to further explore the signaling pathways involving JNK in trophoblast differentiation and see if this result holds true in the IUGR trophoblast cultures.

I showed in this thesis that caspase cleavage was not necessary for trophoblast differentiation. This is in contrast to those results presented by Black and others (16) in the placental explant model. It would be important to confirm my results using the placental explant model and confirm that the presence of the pan-caspase inhibitor (ZVAD) has no effect on trophoblast functional differentiation (hCG production). Also, I was unable to detect caspase-8 cleavage in CT cultures even in the presence of TNFα. This could indicate that caspase-8 is fairly inactive in the villous CT population, or that low levels of activation lead to larger downstream events. Future work in our lab will

focus on the direct inhibition of caspase-8 (with Z-IETD-fmk) as well as further work on the detection of cleavage products of caspase-8 in trophoblast cultures.

Although I show in this work that hCG production and syncytialization appear to be separate processes that can occur independently, I think it would be important to test whether neutralizing antibody to hCG is also able to effect morphologic markers of trophoblast differentiation such as placental alkaline phosphatase expression and CT fusion. Previously, preliminary data of hCG immunohistochemical staining of villous trophoblast cultures indicates that only a minority of cells are actively producing hCG. It would also be interesting to compare whether there were more hCG producing cells in the IUGR trophoblast cultures than in normal trophoblast cultures and if this changes over time in culture.

One of the major limitations of my study was that in studying trophoblast differentiation using highly purified villous CT cultured under standard culture conditions (18% oxygen or hyperoxic for the placenta) on plastic culture dishes, I am looking at a process that may be completely different from the natural *in vivo* situation. I propose that further studies of trophoblast differentiation in our lab be undertaken at normoxic conditions for the placenta (roughly 7 to 8% oxygen). In addition, the influence of other 'contaminating' cell types as well as the presence of extracellular matrix molecules is not addressed in the model I used to study trophoblast differentiation. As such, the placental explant model may serve as an important tool for elucidating the contributions of the different placental cell types and how they may interact to modulate trophoblast differentiation.

CHAPTER 7: REFERENCES

1. **Aboagye-Mathiesen, G., M. Zdravkovic, F. D. Toth, and P. Ebbesen.** 1997. Effects of human trophoblast-induced interferons on the expression of proto-oncogenes c-fms/CSF-1R, EGF-R and c-erbB2 in invasive and non-invasive trophoblast. Placenta **18:**155-61.
2. **Abumaree, M. H., P. R. Stone, and L. W. Chamley.** 2006. The effects of apoptotic, deported human placental trophoblast on macrophages: Possible consequences for pregnancy. J Reprod Immunol.
3. **Adams, J. M., and S. Cory.** 1998. The Bcl-2 protein family: arbiters of cell survival. Science **281:**1322-6.
4. **Adler, R. R., A. K. Ng, and N. S. Rote.** 1995. Monoclonal antiphosphatidylserine antibody inhibits intercellular fusion of the choriocarcinoma line, JAR. Biol Reprod **53:**905-10.
5. **Al-Nasiry, S., B. Spitz, M. Hanssens, C. Luyten, and R. Pijnenborg.** 2006. Differential effects of inducers of syncytialization and apoptosis on BeWo and JEG-3 choriocarcinoma cells. Hum Reprod **21:**193-201.
6. **Armant, D. R., B. A. Kilburn, A. Petkova, S. S. Edwin, Z. M. Duniec-Dmuchowski, H. J. Edwards, R. Romero, and R. E. Leach.** 2006. Human trophoblast survival at low oxygen concentrations requires metalloproteinase-mediated shedding of heparin-binding EGF-like growth factor. Development **133:**751-9.
7. **Baczyk, D., C. Dunk, B. Huppertz, C. Maxwell, F. Reister, D. Giannoulias, and J. C. Kingdom.** 2006. Bi-potential behaviour of cytotrophoblasts in first trimester chorionic villi. Placenta **27:**367-74.
8. **Baczyk, D., A. Satkunaratnam, B. Nait-Oumesmar, B. Huppertz, J. C. Cross, and J. C. Kingdom.** 2004. Complex patterns of GCM1 mRNA and protein in villous and extravillous trophoblast cells of the human placenta. Placenta **25:**553-9.
9. **Barker, D. J., A. R. Bull, C. Osmond, and S. J. Simmonds.** 1990. Fetal and placental size and risk of hypertension in adult life. Bmj **301:**259-62.
10. **Barker, D. J., P. D. Winter, C. Osmond, B. Margetts, and S. J. Simmonds.** 1989. Weight in infancy and death from ischaemic heart disease. Lancet **2:**577-80.
11. **Bartha, J. L., R. Romero-Carmona, and R. Comino-Delgado.** 2003. Inflammatory cytokines in intrauterine growth retardation. Acta Obstet Gynecol Scand **82:**1099-102.
12. **Belisle, S., A. Petit, D. Bellabarba, E. Escher, J. G. Lehoux, and N. Gallo-Payet.** 1989. Ca2+, but not membrane lipid hydrolysis, mediates human chorionic gonadotropin production by luteinizing hormone-releasing hormone in human term placenta. J Clin Endocrinol Metab **69:**117-21.
13. **Benirschke, K., and P. Kaufmann.** 2000. Pathology of the Human Placenta, 4th ed. Springer, New York.

14. **Bevers, E. M., P. Comfurius, D. W. Dekkers, and R. F. Zwaal.** 1999. Lipid translocation across the plasma membrane of mammalian cells. Biochim Biophys Acta **1439:**317-30.
15. **Bjerregaard, B., S. Holck, I. J. Christensen, and L. I. Larsson.** 2006. Syncytin is involved in breast cancer-endothelial cell fusions. Cell Mol Life Sci **63:**1906-11.
16. **Black, S., M. Kadyrov, P. Kaufmann, B. Ugele, N. Emans, and B. Huppertz.** 2004. Syncytial fusion of human trophoblast depends on caspase 8. Cell Death Differ **11:**90-8.
17. **Blaise, S., N. de Parseval, L. Benit, and T. Heidmann.** 2003. Genomewide screening for fusogenic human endogenous retrovirus envelopes identifies syncytin 2, a gene conserved on primate evolution. Proc Natl Acad Sci U S A **100:**13013-8.
18. **Blond, J. L., F. Beseme, L. Duret, O. Bouton, F. Bedin, H. Perron, B. Mandrand, and F. Mallet.** 1999. Molecular characterization and placental expression of HERV-W, a new human endogenous retrovirus family. J Virol **73:**1175-85.
19. **Blond, J. L., D. Lavillette, V. Cheynet, O. Bouton, G. Oriol, S. Chapel-Fernandes, B. Mandrand, F. Mallet, and F. L. Cosset.** 2000. An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor. J Virol **74:**3321-9.
20. **Boyd, J. D., and W. J. Hamilton.** 1966. Electron microscopic observations on the cytotrophoblast contribution to the syncytium in the human placenta. J Anat **100:**535-48.
21. **Boyd, J. D., and W. J. Hamilton.** 1966. Electron microscopic observations on the cytotrophoblast contribution to the syncytium in the human placenta. J Anat **100:**535-48.
22. **Bright, N. A., C. D. Ockleford, and M. Anwar.** 1994. Ontogeny and distribution of Fc gamma receptors in the human placenta. Transport or immune surveillance? J Anat **184 (Pt 2):**297-308.
23. **Brosens, I., H. G. Dixon, and W. B. Robertson.** 1977. Fetal growth retardation and the arteries of the placental bed. Br J Obstet Gynaecol **84:**656-63.
24. **Brosens, I., W. B. Robertson, and H. G. Dixon.** 1967. The physiological response of the vessels of the placental bed to normal pregnancy. J Pathol Bacteriol **93:**569-79.
25. **Brown, L. M., H. A. Lacey, P. N. Baker, and I. P. Crocker.** 2005. E-cadherin in the assessment of aberrant placental cytotrophoblast turnover in pregnancies complicated by pre-eclampsia. Histochem Cell Biol **124:**499-506.
26. **Bulmer, J. N., L. Morrison, and P. M. Johnson.** 1988. Expression of the proliferation markers Ki67 and transferrin receptor by human trophoblast populations. J Reprod Immunol **14:**291-302.
27. **Burgos, M. H., and E. M. Rodriguez.** 1966. Specialized zones in the trophoblast of the human term placenta. Am J Obstet Gynecol **96:**342-56.
28. **Caceres, M., and J. W. Thomas.** 2006. The gene of retroviral origin Syncytin 1 is specific to hominoids and is inactive in Old World monkeys. J Hered **97:**100-6.

29. **Calvo, M. T., A. Romo, J. J. Gutierrez, E. Relano, E. Barrio, and A. Ferrandez Longas.** 2004. Study of genetic expression of intrauterine growth factors IGF-I and EGFR in placental tissue from pregnancies with intrauterine growth retardation. J Pediatr Endocrinol Metab **17 Suppl 3:**445-50.
30. **Camussi, G., E. Albano, C. Tetta, and F. Bussolino.** 1991. The molecular action of tumor necrosis factor-alpha. Eur J Biochem **202:**3-14.
31. **Caniggia, I., H. Mostachfi, J. Winter, M. Gassmann, S. J. Lye, M. Kuliszewski, and M. Post.** 2000. Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3). J Clin Invest **105:**577-87.
32. **Carpentier, I., B. Coornaert, and R. Beyaert.** 2004. Function and regulation of tumor necrosis factor type 2. Curr Med Chem **11:**2205-12.
33. **Carraway, K. L., 3rd, and L. C. Cantley.** 1994. A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. Cell **78:**5-8.
34. **Chaddha, V., S. Viero, B. Huppertz, and J. Kingdom.** 2004. Developmental biology of the placenta and the origins of placental insufficiency. Semin Fetal Neonatal Med **9:**357-69.
35. **Chan, G., D. G. Hemmings, A. D. Yurochko, and L. J. Guilbert.** 2002. Human cytomegalovirus-caused damage to placental trophoblasts mediated by immediate-early gene-induced tumor necrosis factor-alpha. Am J Pathol **161:**1371-81.
36. **Chang, C., P. T. Chen, G. D. Chang, C. J. Huang, and H. Chen.** 2004. Functional characterization of the placental fusogenic membrane protein syncytin. Biol Reprod **71:**1956-62.
37. **Chang, C. W., H. C. Chuang, C. Yu, T. P. Yao, and H. Chen.** 2005. Stimulation of GCMa transcriptional activity by cyclic AMP/protein kinase A signaling is attributed to CBP-mediated acetylation of GCMa. Mol Cell Biol **25:**8401-14.
38. **Chattopadhyay, A., M. Vecchi, Q. Ji, R. Mernaugh, and G. Carpenter.** 1999. The role of individual SH2 domains in mediating association of phospholipase C-gamma1 with the activated EGF receptor. J Biol Chem **274:**26091-7.
39. **Chen, C. P., C. Y. Chen, Y. C. Yang, T. H. Su, and H. Chen.** 2004. Decreased placental GCM1 (glial cells missing) gene expression in pre-eclampsia. Placenta **25:**413-21.
40. **Chuang, H. C., C. W. Chang, G. D. Chang, T. P. Yao, and H. Chen.** 2006. Histone deacetylase 3 binds to and regulates the GCMa transcription factor. Nucleic Acids Res **34:**1459-69.
41. **Cotte, C., G. C. Easty, A. M. Neville, and P. Monaghan.** 1980. Preparation of highly purified cytotrophoblast from human placenta with subsequent modulation to form syncytiotrophoblast in monolayer cultures. In Vitro **16:**639-46.
42. **Coussens, L., T. L. Yang-Feng, Y. C. Liao, E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, and et al.** 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science **230:**1132-9.

43. **Coutifaris, C., L. C. Kao, H. M. Sehdev, U. Chin, G. O. Babalola, O. W. Blaschuk, and J. F. Strauss, 3rd.** 1991. E-cadherin expression during the differentiation of human trophoblasts. Development **113:**767-77.
44. **Cowden Dahl, K. D., B. H. Fryer, F. A. Mack, V. Compernolle, E. Maltepe, D. M. Adelman, P. Carmeliet, and M. C. Simon.** 2005. Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Mol Cell Biol **25:**10479-91.
45. **Crocker, I. P., S. Cooper, S. C. Ong, and P. N. Baker.** 2003. Differences in apoptotic susceptibility of cytotrophoblasts and syncytiotrophoblasts in normal pregnancy to those complicated with preeclampsia and intrauterine growth restriction. Am J Pathol **162:**637-43.
46. **Crocker, I. P., D. M. Tansinda, C. J. Jones, and P. N. Baker.** 2004. The influence of oxygen and tumor necrosis factor-alpha on the cellular kinetics of term placental villous explants in culture. J Histochem Cytochem **52:**749-57.
47. **Cronier, L., J. L. Frendo, N. Defamie, G. Pidoux, G. Bertin, J. Guibourdenche, G. Pointis, and A. Malassine.** 2003. Requirement of gap junctional intercellular communication for human villous trophoblast differentiation. Biol Reprod **69:**1472-80.
48. **Cross, J. C.** 2006. Placental function in development and disease. Reprod Fertil Dev **18:**71-6.
49. **Dagher, Z., G. Garcon, S. Billet, P. Gosset, F. Ledoux, D. Courcot, A. Aboukais, and P. Shirali.** 2006. Activation of different pathways of apoptosis by air pollution particulate matter (PM2.5) in human epithelial lung cells (L132) in culture. Toxicology **225:**12-24.
50. **Damsky, C. H., M. L. Fitzgerald, and S. J. Fisher.** 1992. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. J Clin Invest **89:**210-22.
51. **Daoud, G., M. Amyot, E. Rassart, A. Masse, L. Simoneau, and J. Lafond.** 2005. ERK1/2 and p38 regulate trophoblasts differentiation in human term placenta. J Physiol **566:**409-23.
52. **Daoud, G., E. Rassart, A. Masse, and J. Lafond.** 2006. Src family kinases play multiple roles in differentiation of trophoblasts from human term placenta. J Physiol **571:**537-53.
53. **Das, M., B. Xu, L. Lin, S. Chakrabarti, V. Shivaswamy, and N. S. Rote.** 2004. Phosphatidylserine efflux and intercellular fusion in a BeWo model of human villous cytotrophoblast. Placenta **25:**396-407.
54. **De Falco, M., V. Fedele, L. Cobellis, A. Mastrogiacomo, D. Giraldi, S. Leone, L. De Luca, V. Laforgia, and A. De Luca.** 2004. Pattern of expression of cyclin D1/CDK4 complex in human placenta during gestation. Cell Tissue Res **317:**187-94.
55. **Dempsey, E. W., and S. A. Luse.** 1971. Regional specializations in the syncytial trophoblast of early human placentas. J Anat **108:**545-61.
56. **Desai, J., V. Holt-Shore, R. J. Torry, M. R. Caudle, and D. S. Torry.** 1999. Signal transduction and biological function of placenta growth factor in primary human trophoblast. Biol Reprod **60:**887-92.

57. **Dhar, R., S. Karmakar, R. Sriraman, G. P. Talwar, and C. Das.** 2004. Efficacy of a recombinant chimeric anti-hCG antibody to prevent human cytotrophoblasts fusion and block progesterone synthesis. Am J Reprod Immunol **51:**358-63.
58. **Dinarello, C. A.** 1992. The biology of interleukin-1. Chem Immunol **51:**1-32.
59. **Dinarello, C. A., and R. C. Thompson.** 1991. Blocking IL-1: interleukin 1 receptor antagonist in vivo and in vitro. Immunol Today **12:**404-10.
60. **Eckhart, L., M. Henry, A. M. Santos-Beneit, I. Schmitz, A. Krueger, H. Fischer, J. Bach, J. Ban, S. Kirchhoff, P. H. Krammer, F. Mollinedo, and E. Tschachler.** 2001. Alternative splicing of caspase-8 mRNA during differentiation of human leukocytes. Biochem Biophys Res Commun **289:**777-81.
61. **Erel, C. T., B. Dane, Z. Calay, S. Kaleli, and K. Aydinli.** 2001. Apoptosis in the placenta of pregnancies complicated with IUGR. Int J Gynaecol Obstet **73:**229-35.
62. **Eskenazi, B., L. Fenster, S. Sidney, and E. P. Elkin.** 1993. Fetal growth retardation in infants of multiparous and nulliparous women with preeclampsia. Am J Obstet Gynecol **169:**1112-8.
63. **Formigli, L., L. Papucci, A. Tani, N. Schiavone, A. Tempestini, G. E. Orlandini, S. Capaccioli, and S. Z. Orlandini.** 2000. Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J Cell Physiol **182:**41-9.
64. **Forsythe, J. A., B. H. Jiang, N. V. Iyer, F. Agani, S. W. Leung, R. D. Koos, and G. L. Semenza.** 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol **16:**4604-13.
65. **Frendo, J. L., L. Cronier, G. Bertin, J. Guibourdenche, M. Vidaud, D. Evain-Brion, and A. Malassine.** 2003. Involvement of connexin 43 in human trophoblast cell fusion and differentiation. J Cell Sci **116:**3413-21.
66. **Frendo, J. L., P. Therond, T. Bird, N. Massin, F. Muller, J. Guibourdenche, D. Luton, M. Vidaud, W. B. Anderson, and D. Evain-Brion.** 2001. Overexpression of copper zinc superoxide dismutase impairs human trophoblast cell fusion and differentiation. Endocrinology **142:**3638-48.
67. **Galet, C., and M. Ascoli.** 2005. The differential binding affinities of the luteinizing hormone (LH)/choriogonadotropin receptor for LH and choriogonadotropin are dictated by different extracellular domain residues. Mol Endocrinol **19:**1263-76.
68. **Gamero, A. M., and J. J. Oppenheim.** 2006. IL-1 can act as number one. Immunity **24:**16-7.
69. **Garcia-Lloret, M. I., D. W. Morrish, T. G. Wegmann, L. Honore, A. R. Turner, and L. J. Guilbert.** 1994. Demonstration of functional cytokine-placental interactions: CSF-1 and GM-CSF stimulate human cytotrophoblast differentiation and peptide hormone secretion. Exp Cell Res **214:**46-54.
70. **Garcia-Lloret, M. I., J. Yui, B. Winkler-Lowen, and L. J. Guilbert.** 1996. Epidermal growth factor inhibits cytokine-induced apoptosis of primary human trophoblasts. J Cell Physiol **167:**324-32.
71. **Genbacev, O., R. Joslin, C. H. Damsky, B. M. Polliotti, and S. J. Fisher.** 1996. Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in

vitro and models the placental defects that occur in preeclampsia. J Clin Invest **97:**540-50.

72. **Genbacev, O., Y. Zhou, J. W. Ludlow, and S. J. Fisher.** 1997. Regulation of human placental development by oxygen tension. Science **277:**1669-72.
73. **Getsios, S., and C. D. MacCalman.** 2003. Cadherin-11 modulates the terminal differentiation and fusion of human trophoblastic cells in vitro. Dev Biol **257:**41-54.
74. **Goffin, F., C. Munaut, A. Malassine, D. Evain-Brion, F. Frankenne, V. Fridman, M. Dubois, S. Uzan, P. Merviel, and J. M. Foidart.** 2003. Evidence of a limited contribution of feto-maternal interactions to trophoblast differentiation along the invasive pathway. Tissue Antigens **62:**104-16.
75. **Guilbert, L. J., B. Winkler-Lowen, R. Sherburne, N. S. Rote, H. Li, and D. W. Morrish.** 2002. Preparation and functional characterization of villous cytotrophoblasts free of syncytial fragments. Placenta **23:**175-83.
76. **Hakoshima, T., and K. Tomita.** 1988. Crystallization and preliminary X-ray investigation reveals that tumor necrosis factor is a compact trimer furnished with 3-fold symmetry. J Mol Biol **201:**455-7.
77. **Hamilton, W. J., and J. D. Boyd.** 1966. Specializations of the syncytium of the human chorion. Br Med J **5502:**1501-6.
78. **Harman, I., A. Costello, A. Sane, and S. Handwerger.** 1987. Cyclic adenosine-3',5'-monophosphate stimulates the acute release of placental lactogen from human trophoblast cells. Endocrinology **121:**59-63.
79. **Harris, R. D., W. A. Simpson, L. R. Pet, M. Marin-Padilla, and H. C. Crow.** 1990. Placental hypoechoic-anechoic areas and infarction: sonographic-pathologic correlation. Radiology **176:**75-80.
80. **Heaman, M. I., and K. Chalmers.** 2005. Prevalence and correlates of smoking during pregnancy: a comparison of aboriginal and non-aboriginal women in manitoba. Birth **32:**299-305.
81. **Henriksen, T., and T. Clausen.** 2002. The fetal origins hypothesis: placental insufficiency and inheritance versus maternal malnutrition in well-nourished populations. Acta Obstet Gynecol Scand **81:**112-4.
82. **Hershkovitz, R., J. C. Kingdom, M. Geary, and C. H. Rodeck.** 2000. Fetal cerebral blood flow redistribution in late gestation: identification of compromise in small fetuses with normal umbilical artery Doppler. Ultrasound Obstet Gynecol **15:**209-12.
83. **Heyborne, K. D., S. S. Witkin, and J. A. McGregor.** 1992. Tumor necrosis factor-alpha in midtrimester amniotic fluid is associated with impaired intrauterine fetal growth. Am J Obstet Gynecol **167:**920-5.
84. **Hills, F. A., V. M. Abrahams, B. Gonzalez-Timon, J. Francis, B. Cloke, L. Hinkson, R. Rai, G. Mor, L. Regan, M. Sullivan, E. W. Lam, and J. J. Brosens.** 2006. Heparin prevents programmed cell death in human trophoblast. Mol Hum Reprod **12:**237-243.
85. **Holcberg, G., M. Huleihel, O. Sapir, M. Katz, M. Tsadkin, B. Furman, M. Mazor, and L. Myatt.** 2001. Increased production of tumor necrosis factor-alpha TNF-alpha by IUGR human placentae. Eur J Obstet Gynecol Reprod Biol **94:**69-72.

86. **Huot, R. I., J. M. Foidart, R. M. Nardone, and K. Stromberg.** 1981. Differential modulation of human chorionic gonadotropin secretion by epidermal growth factor in normal and malignant placental cultures. J Clin Endocrinol Metab **53:**1059-63.

87. **Huppertz, B., H. G. Frank, and P. Kaufmann.** 1999. The apoptosis cascade--morphological and immunohistochemical methods for its visualization. Anat Embryol (Berl) **200:**1-18.

88. **Huppertz, B., H. G. Frank, J. C. Kingdom, F. Reister, and P. Kaufmann.** 1998. Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. Histochem Cell Biol **110:**495-508.

89. **Huppertz, B., H. G. Frank, F. Reister, J. Kingdom, H. Korr, and P. Kaufmann.** 1999. Apoptosis cascade progresses during turnover of human trophoblast: analysis of villous cytotrophoblast and syncytial fragments in vitro. Lab Invest **79:**1687-702.

90. **Huppertz, B., M. Kadyrov, and J. C. Kingdom.** 2006. Apoptosis and its role in the trophoblast. Am J Obstet Gynecol.

91. **Huppertz, B., J. Kingdom, I. Caniggia, G. Desoye, S. Black, H. Korr, and P. Kaufmann.** 2003. Hypoxia favours necrotic versus apoptotic shedding of placental syncytiotrophoblast into the maternal circulation. Placenta **24:**181-90.

92. **Huppertz, B., and J. C. Kingdom.** 2004. Apoptosis in the trophoblast--role of apoptosis in placental morphogenesis. J Soc Gynecol Investig **11:**353-62.

93. **Huppertz, B., P. Kaufmann, and J. Kingdom.** 2002. Trophoblast turnover in health and disease. Fetal and Maternal Medicine Review **13:**103-118.

94. **Huppertz, B., D. S. Tews, and P. Kaufmann.** 2001. Apoptosis and syncytial fusion in human placental trophoblast and skeletal muscle. Int Rev Cytol **205:**215-53.

95. **Ishihara, N., H. Matsuo, H. Murakoshi, J. B. Laoag-Fernandez, T. Samoto, and T. Maruo.** 2002. Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation. Am J Obstet Gynecol **186:**158-66.

96. **Itoh, N., and S. Nagata.** 1993. A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen. J Biol Chem **268:**10932-7.

97. **Jiang, K., Y. Chen, and J. N. Jarvis.** 2006. hCG Secretion in human choriocarcinoma JAR cells is MAPK but not Stat3 dependent: contributions of TNFalpha and IL-1beta to inflammation-induced hCG secretion. Placenta **27:**853-60.

98. **Jimenez, V., M. Henriquez, P. Llanos, and G. Riquelme.** 2004. Isolation and purification of human placental plasma membranes from normal and pre-eclamptic pregnancies. a comparative study. Placenta **25:**422-37.

99. **Johnstone, E. D., G. Chan, C. P. Sibley, S. T. Davidge, B. Lowen, and L. J. Guilbert.** 2005. Sphingosine-1-phosphate inhibition of placental trophoblast differentiation through a G(i)-coupled receptor response. J Lipid Res **46:**1833-9.

100. **Johnstone, E. D., M. Mackova, S. Das, S. G. Payne, B. Lowen, C. P. Sibley, G. Chan, and L. J. Guilbert.** 2005. Multiple anti-apoptotic pathways stimulated by EGF in cytotrophoblasts. Placenta **26:**548-55.

101. **Johnstone, E. D., C. P. Sibley, B. Lowen, and L. J. Guilbert.** 2005. Epidermal Growth Factor Stimulation of Trophoblast Differentiation Requires MAPK11/14 (p38 MAP Kinase) Activation. Biol Reprod **73:**1282-8.
102. **Jokhi, P. P., A. King, and Y. W. Loke.** 1994. Reciprocal expression of epidermal growth factor receptor (EGF-R) and c-erbB2 by non-invasive and invasive human trophoblast populations. Cytokine **6:**433-42.
103. **Jones, C. J., and H. Fox.** 1977. Syncytial knots and intervillous bridges in the human placenta: an ultrastructural study. J Anat **124:**275-86.
104. **Jorissen, R. N., F. Walker, N. Pouliot, T. P. Garrett, C. W. Ward, and A. W. Burgess.** 2003. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res **284:**31-53.
105. **Jurisicova, A., J. Detmar, and I. Caniggia.** 2005. Molecular mechanisms of trophoblast survival: from implantation to birth. Birth Defects Res C Embryo Today **75:**262-80.
106. **Ka, H., and J. S. Hunt.** 2006. FLICE-inhibitory protein: expression in early and late gestation human placentas. Placenta **27:**626-34.
107. **Ka, H., and J. S. Hunt.** 2003. Temporal and spatial patterns of expression of inhibitors of apoptosis in human placentas. Am J Pathol **163:**413-22.
108. **Kadyrov, M., P. Kaufmann, and B. Huppertz.** 2001. Expression of a cytokeratin 18 neo-epitope is a specific marker for trophoblast apoptosis in human placenta. Placenta **22:**44-8.
109. **Kameda, T., N. Matsuzaki, K. Sawai, T. Okada, F. Saji, T. Matsuda, T. Hirano, T. Kishimoto, and O. Tanizawa.** 1990. Production of interleukin-6 by normal human trophoblast. Placenta **11:**205-13.
110. **Kao, L. C., S. Caltabiano, S. Wu, J. F. Strauss, 3rd, and H. J. Kliman.** 1988. The human villous cytotrophoblast: interactions with extracellular matrix proteins, endocrine function, and cytoplasmic differentiation in the absence of syncytium formation. Dev Biol **130:**693-702.
111. **Karmakar, S., and C. Das.** 2004. Modulation of ezrin and E-cadherin expression by IL-1beta and TGF-beta1 in human trophoblasts. J Reprod Immunol **64:**9-29.
112. **Katsuragawa, H., H. Kanzaki, T. Inoue, T. Hirano, T. Mori, and N. S. Rote.** 1997. Monoclonal antibody against phosphatidylserine inhibits in vitro human trophoblastic hormone production and invasion. Biol Reprod **56:**50-8.
113. **Kauma, S. W., T. T. Turner, and J. R. Harty.** 1994. Interleukin-1 beta stimulates interleukin-6 production in placental villous core mesenchymal cells. Endocrinology **134:**457-60.
114. **Keryer, G., E. Alsat, K. Tasken, and D. Evain-Brion.** 1998. Cyclic AMP-dependent protein kinases and human trophoblast cell differentiation in vitro. J Cell Sci **111 (Pt 7):**995-1004.
115. **Kilani, R. T., M. Mackova, S. T. Davidge, and L. J. Guilbert.** 2003. Effect of oxygen levels in villous trophoblast apoptosis. Placenta **24:**826-34.
116. **Kingdom, J., and P. N. Baker.** 2000. Intrauterine Growth Restriction, 1st ed. Springer-Verlag, London.
117. **Kingdom, J. C., and P. Kaufmann.** 1997. Oxygen and placental villous development: origins of fetal hypoxia. Placenta **18:**613-21; discussion 623-6.

118. **Kliman, H. J., J. E. Nestler, E. Sermasi, J. M. Sanger, and J. F. Strauss, 3rd.** 1986. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology **118:**1567-82.
119. **Klonisch, T., P. Wolf, S. Hombach-Klonisch, S. Vogt, A. Kuechenhoff, F. Tetens, and B. Fischer.** 2001. Epidermal growth factor-like ligands and erbB genes in the peri-implantation rabbit uterus and blastocyst. Biol Reprod **64:**1835-44.
120. **Knerr, I., E. Beinder, and W. Rascher.** 2002. Syncytin, a novel human endogenous retroviral gene in human placenta: evidence for its dysregulation in preeclampsia and HELLP syndrome. Am J Obstet Gynecol **186:**210-3.
121. **Knerr, I., B. Huppertz, C. Weigel, J. Dotsch, C. Wich, R. L. Schild, M. W. Beckmann, and W. Rascher.** 2004. Endogenous retroviral syncytin: compilation of experimental research on syncytin and its possible role in normal and disturbed human placentogenesis. Mol Hum Reprod **10:**581-8.
122. **Knerr, I., S. W. Schubert, C. Wich, K. Amann, T. Aigner, T. Vogler, R. Jung, J. Dotsch, W. Rascher, and S. Hashemolhosseini.** 2005. Stimulation of GCMa and syncytin via cAMP mediated PKA signaling in human trophoblastic cells under normoxic and hypoxic conditions. FEBS Lett **579:**3991-8.
123. **Knofler, M., B. Mosl, S. Bauer, G. Griesinger, and P. Husslein.** 2000. TNF-alpha/TNFRI in primary and immortalized first trimester cytotrophoblasts. Placenta **21:**525-35.
124. **Knofler, M., L. Saleh, S. Bauer, B. Galos, H. Rotheneder, P. Husslein, and H. Helmer.** 2004. Transcriptional regulation of the human chorionic gonadotropin beta gene during villous trophoblast differentiation. Endocrinology **145:**1685-94.
125. **Knofler, M., L. Saleh, H. Strohmer, P. Husslein, and M. F. Wolschek.** 1999. Cyclic AMP- and differentiation-dependent regulation of the proximal alphaHCG gene promoter in term villous trophoblasts. Mol Hum Reprod **5:**573-80.
126. **Kudo, Y., C. A. Boyd, I. L. Sargent, and C. W. Redman.** 2003. Hypoxia alters expression and function of syncytin and its receptor during trophoblast cell fusion of human placental BeWo cells: implications for impaired trophoblast syncytialisation in pre-eclampsia. Biochim Biophys Acta **1638:**63-71.
127. **Lambot, N., P. Lebrun, C. Delporte, C. De Vriese, J. Delogne-Desnoeck, A. M. Vanbellinghen, G. Graff, and S. Meuris.** 2005. Effect of IPs, cAMP, and cGMP on the hPL and hCG secretion from human term placenta. Mol Cell Endocrinol **243:**80-5.
128. **Leach, R. E., R. Romero, Y. M. Kim, T. Chaiworapongsa, B. Kilburn, S. K. Das, S. K. Dey, A. Johnson, F. Qureshi, S. Jacques, and D. R. Armant.** 2002. Pre-eclampsia and expression of heparin-binding EGF-like growth factor. Lancet **360:**1215-9.
129. **LeBrun, D. P., R. A. Warnke, and M. L. Cleary.** 1993. Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. Am J Pathol **142:**743-53.
130. **Lee, X., J. C. Keith, Jr., N. Stumm, I. Moutsatsos, J. M. McCoy, C. P. Crum, D. Genest, D. Chin, C. Ehrenfels, R. Pijnenborg, F. A. van Assche, and S. Mi.** 2001. Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia. Placenta **22:**808-12.

131. **Leisser, C., L. Saleh, S. Haider, H. Husslein, S. Sonderegger, and M. Knofler.** 2006. Tumour necrosis factor-{alpha} impairs chorionic gonadotrophin {beta}-subunit expression and cell fusion of human villous cytotrophoblast. Mol Hum Reprod.

132. **Levy, R., S. D. Smith, K. Yusuf, P. C. Huettner, F. T. Kraus, Y. Sadovsky, and D. M. Nelson.** 2002. Trophoblast apoptosis from pregnancies complicated by fetal growth restriction is associated with enhanced p53 expression. Am J Obstet Gynecol **186:**1056-61.

133. **Li, H., J. Dakour, L. J. Guilbert, B. Winkler-Lowen, F. Lyall, and D. W. Morrish.** 2005. PL74, a novel member of the transforming growth factor-beta superfamily, is overexpressed in preeclampsia and causes apoptosis in trophoblast cells. J Clin Endocrinol Metab **90:**3045-53.

134. **Li, H., H. Zhu, C. J. Xu, and J. Yuan.** 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell **94:**491-501.

135. **Li, H. W., A. N. Cheung, S. W. Tsao, A. L. Cheung, and W. S. O.** 2003. Expression of e-cadherin and beta-catenin in trophoblastic tissue in normal and pathological pregnancies. Int J Gynecol Pathol **22:**63-70.

136. **Li, R. H., and L. Z. Zhuang.** 1997. The effects of growth factors on human normal placental cytotrophoblast cell proliferation. Hum Reprod **12:**830-4.

137. **Li, Y., N. Matsuzaki, K. Masuhiro, T. Kameda, T. Taniguchi, F. Saji, K. Yone, and O. Tanizawa.** 1992. Trophoblast-derived tumor necrosis factor-alpha induces release of human chorionic gonadotropin using interleukin-6 (IL-6) and IL-6-receptor-dependent system in the normal human trophoblasts. J Clin Endocrinol Metab **74:**184-91.

138. **Licht, P., V. Russu, and L. Wildt.** 2001. On the role of human chorionic gonadotropin (hCG) in the embryo-endometrial microenvironment: implications for differentiation and implantation. Semin Reprod Med **19:**37-47.

139. **Luttrell, D. K., L. M. Luttrell, and S. J. Parsons.** 1988. Augmented mitogenic responsiveness to epidermal growth factor in murine fibroblasts that overexpress pp60c-src. Mol Cell Biol **8:**497-501.

140. **MacEwan, D. J.** 2002. TNF receptor subtype signalling: differences and cellular consequences. Cell Signal **14:**477-92.

141. **Mackova, M., R. T. Kilani, S. T. Davidge, and L. J. Guilbert.** 2003. The effect of oxygen tension on intracellular survival signalling in primary villous trophoblasts. Placenta **24:**627-37.

142. **Malassine, A., K. Handschuh, V. Tsatsaris, P. Gerbaud, V. Cheynet, G. Oriol, F. Mallet, and D. Evain-Brion.** 2005. Expression of HERV-W Env glycoprotein (syncytin) in the extravillous trophoblast of first trimester human placenta. Placenta **26:**556-62.

143. **Maruo, T., Y. Ashitaka, M. Mochizuki, and S. Tojo.** 1974. Chorionic gonadotropin synthesized in cultivated trophoblast. Endocrinol Jpn **21:**499-505.

144. **Maruo, T., N. Ishihara, T. Samoto, H. Murakoshi, J. B. Laoag-Fernandez, and H. Matsuo.** 2001. Regulation of human trophoblast proliferation and apoptosis during pregnancy. Early Pregnancy **5:**28-9.

145. **Maruo, T., H. Matsuo, T. Oishi, M. Hayashi, R. Nishino, and M. Mochizuki.** 1987. Induction of differentiated trophoblast function by epidermal growth factor: relation of immunohistochemically detected cellular epidermal growth factor receptor levels. J Clin Endocrinol Metab **64:**744-50.

146. **Maruo, T., H. Matsuo, T. Otani, and M. Mochizuki.** 1995. Role of epidermal growth factor (EGF) and its receptor in the development of the human placenta. Reprod Fertil Dev **7:**1465-70.

147. **Masuhiro, K., N. Matsuzaki, E. Nishino, T. Taniguchi, T. Kameda, Y. Li, F. Saji, and O. Tanizawa.** 1991. Trophoblast-derived interleukin-1 (IL-1) stimulates the release of human chorionic gonadotropin by activating IL-6 and IL-6-receptor system in first trimester human trophoblasts. J Clin Endocrinol Metab **72:**594-601.

148. **Matouskova, M., J. Blazkova, P. Pajer, A. Pavlicek, and J. Hejnar.** 2006. CpG methylation suppresses transcriptional activity of human syncytin-1 in non-placental tissues. Exp Cell Res **312:**1011-20.

149. **Mayhew, T. M., L. Leach, R. McGee, W. W. Ismail, R. Myklebust, and M. J. Lammiman.** 1999. Proliferation, differentiation and apoptosis in villous trophoblast at 13-41 weeks of gestation (including observations on annulate lamellae and nuclear pore complexes). Placenta **20:**407-22.

150. **Medema, J. P., C. Scaffidi, F. C. Kischkel, A. Shevchenko, M. Mann, P. H. Krammer, and M. E. Peter.** 1997. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). Embo J **16:**2794-804.

151. **Melillo, G., T. Musso, A. Sica, L. S. Taylor, G. W. Cox, and L. Varesio.** 1995. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. J Exp Med **182:**1683-93.

152. **Mi, S., X. Lee, X. Li, G. M. Veldman, H. Finnerty, L. Racie, E. LaVallie, X. Y. Tang, P. Edouard, S. Howes, J. C. Keith, Jr., and J. M. McCoy.** 2000. Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. Nature **403:**785-9.

153. **Miller, M. J., C. A. Voelker, S. Olister, J. H. Thompson, X. J. Zhang, D. Rivera, S. Eloby-Childress, X. Liu, D. A. Clark, and M. R. Pierce.** 1996. Fetal growth retardation in rats may result from apoptosis: role of peroxynitrite. Free Radic Biol Med **21:**619-29.

154. **Miyashita, T., and J. C. Reed.** 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell **80:**293-9.

155. **Monzon-Bordonaba, F., F. Vadillo-Ortega, and R. F. Feinberg.** 2002. Modulation of trophoblast function by tumor necrosis factor-alpha: a role in pregnancy establishment and maintenance? Am J Obstet Gynecol **187:**1574-80.

156. **Morrish, D. W., J. Dakour, and H. Li.** 1998. Functional regulation of human trophoblast differentiation. J Reprod Immunol **39:**179-95.

157. **Morrish, D. W., J. Dakour, and H. Li.** 2001. Life and death in the placenta: new peptides and genes regulating human syncytiotrophoblast and extravillous cytotrophoblast lineage formation and renewal. Curr Protein Pept Sci **2:**245-59.

158. **Morrish, D. W., J. Dakour, H. Li, J. Xiao, R. Miller, R. Sherburne, R. C. Berdan, and L. J. Guilbert.** 1997. In vitro cultured human term cytotrophoblast: a model for normal primary epithelial cells demonstrating a spontaneous

differentiation programme that requires EGF for extensive development of syncytium. Placenta **18:**577-85.

159. **Muir, A., A. M. Lever, and A. Moffett.** 2006. Human endogenous retrovirus-W envelope (syncytin) is expressed in both villous and extravillous trophoblast populations. J Gen Virol **87:**2067-71.
160. **Myatt, L., and X. Cui.** 2004. Oxidative stress in the placenta. Histochem Cell Biol **122:**369-82.
161. **Nelson, D. M.** 1996. Apoptotic changes occur in syncytiotrophoblast of human placental villi where fibrin type fibrinoid is deposited at discontinuities in the villous trophoblast. Placenta **17:**387-91.
162. **Ness, R. B., and B. M. Sibai.** 2006. Shared and disparate components of the pathophysiologies of fetal growth restriction and preeclampsia. Am J Obstet Gynecol **195:**40-9.
163. **Nevo, O., N. Soleymanlou, Y. Wu, J. Xu, J. Kingdom, A. Many, S. Zamudio, and I. Caniggia.** 2006. Increased Expression of sFlt-1 in In Vivo and In Vitro Models of Human Placental Hypoxia is Mediated by HIF-1. Am J Physiol Regul Integr Comp Physiol.
164. **Newby, D., L. Marks, F. Cousins, E. Duffie, and F. Lyall.** 2005. Villous explant culture: characterization and evaluation of a model to study trophoblast invasion. Hypertens Pregnancy **24:**75-91.
165. **Nishino, E., N. Matsuzaki, K. Masuhiro, T. Kameda, T. Taniguchi, T. Takagi, F. Saji, and O. Tanizawa.** 1990. Trophoblast-derived interleukin-6 (IL-6) regulates human chorionic gonadotropin release through IL-6 receptor on human trophoblasts. J Clin Endocrinol Metab **71:**436-41.
166. **Nulsen, J. C., M. J. Woolkalis, G. S. Kopf, and J. F. Strauss, 3rd.** 1988. Adenylate cyclase in human cytotrophoblasts: characterization and its role in modulating human chorionic gonadotropin secretion. J Clin Endocrinol Metab **66:**258-65.
167. **Pardi, G., I. Cetin, A. M. Marconi, P. Bozzetti, M. Buscaglia, E. L. Makowski, and F. C. Battaglia.** 1992. Venous drainage of the human uterus: respiratory gas studies in normal and fetal growth-retarded pregnancies. Am J Obstet Gynecol **166:**699-706.
168. **Payne, S. G., S. C. Smith, S. T. Davidge, P. N. Baker, and L. J. Guilbert.** 1999. Death receptor Fas/Apo-1/CD95 expressed by human placental cytotrophoblasts does not mediate apoptosis. Biol Reprod **60:**1144-50.
169. **Petit, A., N. Gallo-Payet, D. Bellabarba, J. G. Lehoux, and S. Belisle.** 1993. The modulation of placental lactogen release by opioids: a role for extracellular calcium. Mol Cell Endocrinol **90:**165-70.
170. **Petroff, M. G.** 2005. Immune interactions at the maternal-fetal interface. J Reprod Immunol **68:**1-13.
171. **Pijnenborg, R., C. Luyten, L. Vercruysse, J. C. Keith, Jr., and F. A. Van Assche.** 2000. Cytotoxic effects of tumour necrosis factor (TNF)-alpha and interferon-gamma on cultured human trophoblast are modulated by fibronectin. Mol Hum Reprod **6:**635-41.
172. **Polette, M., N. Gilbert, I. Stas, B. Nawrocki, A. Noel, A. Remacle, W. G. Stetler-Stevenson, P. Birembaut, and M. Foidart.** 1994. Gelatinase A

expression and localization in human breast cancers. An in situ hybridization study and immunohistochemical detection using confocal microscopy. Virchows Arch **424:**641-5.

173. **Potgens, A. J., S. Drewlo, M. Kokozidou, and P. Kaufmann.** 2004. Syncytin: the major regulator of trophoblast fusion? Recent developments and hypotheses on its action. Hum Reprod Update **10:**487-96.
174. **Prenzel, N., O. M. Fischer, S. Streit, S. Hart, and A. Ullrich.** 2001. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr Relat Cancer **8:**11-31.
175. **Richart, R.** 1961. Studies of placental morphogenesis. I. Radioautographic studies of human placenta utilizing tritiated thymidine. Proc Soc Exp Biol Med **106:**829-31.
176. **Roberts, J. M., and H. S. Gammill.** 2005. Preeclampsia: recent insights. Hypertension **46:**1243-9.
177. **Roberts, J. M., and K. Y. Lain.** 2002. Recent Insights into the pathogenesis of pre-eclampsia. Placenta **23:**359-72.
178. **Robinson, J. S., and J. A. Owens.** 1996. Pathophysiology of intrauterine growth failure. Arnold, London.
179. **Rodesch, F., P. Simon, C. Donner, and E. Jauniaux.** 1992. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet Gynecol **80:**283-5.
180. **Rodway, M., F. Z. Zhou, J. Benoit, B. H. Yuen, and P. C. Leung.** 1988. Differential effects of 8-bromo-cyclic AMP on human chorionic gonadotropin (hCG), progesterone and estrogen production by term placental cells. Life Sci **43:**1451-8.
181. **Sakuragi, N., H. Matsuo, G. Coukos, E. E. Furth, M. P. Bronner, C. M. VanArsdale, S. Krajewsky, J. C. Reed, and J. F. Strauss, 3rd.** 1994. Differentiation-dependent expression of the BCL-2 proto-oncogene in the human trophoblast lineage. J Soc Gynecol Investig **1:**164-72.
182. **Sanders, E. J., and E. Parker.** 2002. The role of mitochondria, cytochrome c and caspase-9 in embryonic lens fibre cell denucleation. J Anat **201:**121-35.
183. **Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K. J. Tomaselli, K. M. Debatin, P. H. Krammer, and M. E. Peter.** 1998. Two CD95 (APO-1/Fas) signaling pathways. Embo J **17:**1675-87.
184. **Secchiero, P., A. Gonelli, P. Mirandola, E. Melloni, L. Zamai, C. Celeghini, D. Milani, and G. Zauli.** 2002. Tumor necrosis factor-related apoptosis-inducing ligand induces monocytic maturation of leukemic and normal myeloid precursors through a caspase-dependent pathway. Blood **100:**2421-9.
185. **Semenza, G. L., P. H. Roth, H. M. Fang, and G. L. Wang.** 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem **269:**23757-63.
186. **Silva, C. M.** 2004. Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. Oncogene **23:**8017-23.
187. **Siman, C. M., C. P. Sibley, C. J. Jones, M. A. Turner, and S. L. Greenwood.** 2001. The functional regeneration of syncytiotrophoblast in cultured explants of term placenta. Am J Physiol Regul Integr Comp Physiol **280:**R1116-22.

188. **Simon, C., A. Frances, G. Piquette, M. Hendrickson, A. Milki, and M. L. Polan.** 1994. Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine/paracrine function. J Clin Endocrinol Metab **78:**847-54.

189. **Simpson, R. A., T. M. Mayhew, and P. R. Barnes.** 1992. From 13 weeks to term, the trophoblast of human placenta grows by the continuous recruitment of new proliferative units: a study of nuclear number using the disector. Placenta **13:**501-12.

190. **Smith, S. C., P. N. Baker, and E. M. Symonds.** 1997. Apoptosis within the trophoblast: its distinctive electron microscopy features, and the role which it plays in the pathophysiology of intrauterine growth restriction. J Soc Gynecol Invest **4:**95A.

191. **Smith, S. C., P. N. Baker, and E. M. Symonds.** 1997. Increased placental apoptosis in intrauterine growth restriction. Am J Obstet Gynecol **177:**1395-401.

192. **Soltoff, S. P., K. L. Carraway, 3rd, S. A. Prigent, W. G. Gullick, and L. C. Cantley.** 1994. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol Cell Biol **14:**3550-8.

193. **Srisuparp, S., Z. Strakova, and A. T. Fazleabas.** 2001. The role of chorionic gonadotropin (CG) in blastocyst implantation. Arch Med Res **32:**627-34.

194. **Stallmach, T., G. Hebisch, H. Joller, P. Kolditz, and M. Engelmann.** 1995. Expression pattern of cytokines in the different compartments of the feto-maternal unit under various conditions. Reprod Fertil Dev **7:**1573-80.

195. **Stephanou, A., and S. Handwerger.** 1994. Interleukin-6 stimulates placental lactogen expression by human trophoblast cells. Endocrinology **135:**719-23.

196. **Straszewski-Chavez, S. L., V. M. Abrahams, E. F. Funai, and G. Mor.** 2004. X-linked inhibitor of apoptosis (XIAP) confers human trophoblast cell resistance to Fas-mediated apoptosis. Mol Hum Reprod **10:**33-41.

197. **Straszewski-Chavez, S. L., V. M. Abrahams, and G. Mor.** 2005. The role of apoptosis in the regulation of trophoblast survival and differentiation during pregnancy. Endocr Rev **26:**877-97.

198. **Tan, T. Y., and G. S. Yeo.** 2005. Intrauterine growth restriction. Curr Opin Obstet Gynecol **17:**135-42.

199. **Tsujimoto, Y.** 2003. Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol **195:**158-67.

200. **Tsukihara, S., T. Harada, I. Deura, M. Mitsunari, S. Yoshida, T. Iwabe, and N. Terakawa.** 2004. Interleukin-1beta-induced expression of IL-6 and production of human chorionic gonadotropin in human trophoblast cells via nuclear factor-kappaB activation. Am J Reprod Immunol **52:**218-23.

201. **Viero, S., V. Chaddha, F. Alkazaleh, M. J. Simchen, A. Malik, E. Kelly, R. Windrim, and J. C. Kingdom.** 2004. Prognostic value of placental ultrasound in pregnancies complicated by absent end-diastolic flow velocity in the umbilical arteries. Placenta **25:**735-41.

202. **Villar, J., G. Carroli, D. Wojdyla, E. Abalos, D. Giordano, H. Ba'aqeel, U. Farnot, P. Bergsjo, L. Bakketeig, P. Lumbiganon, L. Campodonico, Y. Al-Mazrou, M. Lindheimer, and M. Kramer.** 2006. Preeclampsia, gestational

hypertension and intrauterine growth restriction, related or independent conditions? Am J Obstet Gynecol **194:**921-31.

203. **Walker, J. J.** 2000. Pre-eclampsia. Lancet **356:**1260-1265.
204. **Watanabe, H.** 1990. Epidermal growth factor in urine of pregnant women and in amniotic fluid throughout pregnancy. Gynecol Endocrinol **4:**43-50.
205. **Wilcox, A. J.** 1983. Intrauterine growth retardation: beyond birthweight criteria. Early Hum Dev **8:**189-93.
206. **Wong, R. W., and L. Guillaud.** 2004. The role of epidermal growth factor and its receptors in mammalian CNS. Cytokine Growth Factor Rev **15:**147-56.
207. **Yamada, M., T. Ikeuchi, and H. Hatanaka.** 1997. The neurotrophic action and signalling of epidermal growth factor. Prog Neurobiol **51:**19-37.
208. **Yamada, Z., M. Kitagawa, T. Takemura, and K. Hirokawa.** 2001. Effect of maternal age on incidences of apoptotic and proliferative cells in trophoblasts of full-term human placenta. Mol Hum Reprod **7:**1179-85.
209. **Yang, M., Z. M. Lei, and V. Rao Ch.** 2003. The central role of human chorionic gonadotropin in the formation of human placental syncytium. Endocrinology **144:**1108-20.
210. **Yang, Y., K. K. Yelavarthi, H. L. Chen, J. L. Pace, P. F. Terranova, and J. S. Hunt.** 1993. Molecular, biochemical, and functional characteristics of tumor necrosis factor-alpha produced by human placental cytotrophoblastic cells. J Immunol **150:**5614-24.
211. **Yu, C., K. Shen, M. Lin, P. Chen, C. Lin, G. D. Chang, and H. Chen.** 2002. GCMa regulates the syncytin-mediated trophoblastic fusion. J Biol Chem **277:**50062-8.
212. **Yui, J., M. Garcia-Lloret, A. J. Brown, R. C. Berdan, D. W. Morrish, T. G. Wegmann, and L. J. Guilbert.** 1994. Functional, long-term cultures of human term trophoblasts purified by column-elimination of CD9 expressing cells. Placenta **15:**231-46.
213. **Yui, J., M. Garcia-Lloret, T. G. Wegmann, and L. J. Guilbert.** 1994. Cytotoxicity of tumour necrosis factor-alpha and gamma-interferon against primary human placental trophoblasts. Placenta **15:**819-35.
214. **Yui, J., D. Hemmings, M. Garcia-Lloret, and L. J. Guilbert.** 1996. Expression of the human p55 and p75 tumor necrosis factor receptors in primary villous trophoblasts and their role in cytotoxic signal transduction. Biol Reprod **55:**400-9.
215. **Yusuf, K., S. D. Smith, Y. Sadovsky, and D. M. Nelson.** 2002. Trophoblast differentiation modulates the activity of caspases in primary cultures of term human trophoblasts. Pediatr Res **52:**411-5.